IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

SYNGENTA SEEDS, INC., GOLDEN HARVEST SEEDS, INC., and GARST SEED COMPANY, Plaintiffs, v. DEKALB GENETICS CORPORATION, Defendant.	Civil Action No.:

DECLARATORY JUDGMENT COMPLAINT

Plaintiffs Syngenta Seeds, Inc., Golden Harvest Seeds, Inc., and Garst Seed Company (collectively "Syngenta"), by its undersigned counsel, bring this declaratory judgment action against Defendant DeKalb Genetics Corporation ("DeKalb"). Syngenta alleges as follows:

JURISDICTION AND VENUE

1. Syngenta brings this civil action under the Patent Laws of the United States, 35 U.S.C. § 1 et seq., and the Declaratory Judgment Act, 28 U.S.C. §§ 2201 and 2202, to obtain a declaration that U.S. Patent No. 6,946,587 ("the Lundquist '587 patent") and U.S. Patent No. 5,538,877 ("the Lundquist '877 patent"), both assigned to DeKalb, are not infringed by Syngenta and/or are invalid. True and correct copies of the Lundquist '587 and '877 patents are attached as Exhibits A and B, respectively.

2. This Court has subject matter jurisdiction over this action under 28 U.S.C. §§ 1331 and 1338(a).

3. This Court has personal jurisdiction over DeKalb, a Delaware corporation.

4. Venue is proper in this judicial district under 28 U.S.C. §§ 1391(b) and (c).

THE PARTIES

5. Syngenta Seeds, Inc. is a corporation duly organized under the laws of the State of Delaware with its principal place of business at 7500 Olson Memorial Highway, Golden Valley, Minnesota 55427.

6. Golden Harvest Seeds, Inc. is a corporation duly organized under the laws of the State of Delaware with its principal place of business at 100 JC Robinson Boulevard, Waterloo, Nebraska 68069.

7. Garst Seed Company is a corporation duly organized under the laws of the State of Delaware with its principal place of business at 2369 330th Street, Slater, Iowa 50244.

8. On information and belief, DeKalb is a corporation duly organized under the laws of the State of Delaware with a principal place of business at 3100 Sycamore Road, DeKalb, Illinois 60115.

9. DeKalb is a wholly owned subsidiary of Monsanto Company ("Monsanto"). Monsanto is a corporation duly organized under the laws of the State of Delaware with a principal place of business at 800 North Lindbergh Boulevard, St. Louis, Missouri 63167.

FACTUAL BACKGROUND

A. Corn Seeds and Traits

10. Corn is the leading cash crop in the United States, with an annual value of over $24 billion. In 2005, approximately 81 million acres of corn were grown in the United States.

11. Like most crops, corn production can be impaired by weeds. To control weeds, growers apply herbicides, which are chemical compounds that destroy or inhibit the growth of undesirable plants.

12. "Selective herbicides" are tolerated by the crop but kill or suppress one or more weeds that infest the field. "Non-selective herbicides" are active on all vegetation and do not

distinguish between the commercial crop (such as corn) and other vegetation (such as weeds). Glyphosate is the leading non-selective herbicide used by growers.

13. Biotechnology has made it possible to introduce new genetic characteristics into plant seeds. This is accomplished by transforming the genetic make-up (or "genome") of a seed by inserting new genetic information into the seed genome. The result is called a transgenic "event." An event contains, among other things, a specific gene that expresses a desirable characteristic in the seed.

14. The insertion of a desirable transgenic event into a seed alters the seed's genome, conferring a desirable characteristic, or "trait," on crops grown from the seed.

15. Since 1996, there have been a number of commercially available biotechnology traits for corn seeds. One of the most common of these traits is for glyphosate tolerance. Glyphosate-tolerant corn is resistant to the effect of glyphosate. As a result of this biotechnology, growers that plant glyphosate-tolerant corn may apply glyphosate over an entire field, killing the weeds while allowing the corn crop to survive.

16. Crop protection traits, including herbicide tolerance, are useful to growers because they reduce production costs and increase yield. U.S. farmers are increasingly planting genetically modified varieties of seed. It is estimated that 52% of the corn planted in the U.S. in 2005 was transgenic corn (up from 40% in 2003).

B. Syngenta's Commercialization of GA21 Corn

17. Syngenta is a world-leading agribusiness committed to sustainable agriculture through innovative research and technology. Syngenta is a leader in the commercial seeds market. Syngenta develops and produces a wide range of agricultural products, including biotechnology traits and seeds. Syngenta markets a broad product portfolio of seeds, corn hybrids, and traits in the United States.

18. This case concerns a glyphosate-tolerant transgenic corn line known as "GA21" corn. GA21 corn was created by DeKalb in 1993-1994 using a chimeric gene that had been developed by Rhone-Poulenc Agro ("RPA"). In prior litigation in the Middle District of North Carolina, DeKalb was found liable for intentional fraud, patent infringement, and trade secret misappropriation in connection with its unlawful development and marketing of GA21 corn. As a result of that district court decision, which was affirmed on appeal by the United States Court of Appeals for the Federal Circuit, DeKalb and Monsanto lost their right to market GA21 corn and were forced to commercialize a different glyphosate-tolerant corn event known as "NK603."

19. On February 5, 2004, an affiliate of Syngenta acquired RPA's intellectual property rights to the GA21 glyphosate-tolerant corn trait from RPA's successor, Bayer CropScience. With the acquisition of RPA's rights in GA21 corn, Syngenta has the right to introduce and market its own GA21 corn products and to license others. As part of that acquisition, Syngenta's affiliate also acquired the GA21 license from Bayer CropScience under which Monsanto and its sublicensees operated to produce GA21 corn seeds through 2004.

20. On May 12, 2004, Syngenta's affiliate announced its intention to acquire Advanta BV, including Advanta's Garst Seed subsidiary and its U.S. corn and soybean seed business. At the same time, Syngenta's affiliate publicly announced its acquisition of the GA21 rights from Bayer CropScience.

21. On June 25, 2004, Syngenta announced an agreement to acquire the Golden Harvest group of seed companies. Golden Harvest is a producer of hybrid corn seed.

22. Syngenta's acquisitions of Golden Harvest and Advanta were completed on August 2, 2004, and September 1, 2004, respectively.

23. The combination of the Advanta, Golden Harvest, and GA21 acquisitions has given Syngenta the necessary assets to expand its corn product offerings and to offer seed companies and growers a full range of biotechnology input traits for corn.

24. Syngenta began selling GA21 corn seed in the United States during the 2005 growing season.

25. Syngenta plans to continue to commercialize the GA21 corn trait in its NK®, Golden Harvest®, and Garst® brand seeds, and through licenses to other seed companies.

C. DeKalb's Activities

26. On information and belief, DeKalb is engaged in the business of developing and selling crop seeds in Delaware and elsewhere in the United States.

27. On information and belief, Monsanto, the parent company of DeKalb, sells the herbicide glyphosate under the trademark Roundup® in Delaware and elsewhere in the United States.

28. On information and belief, genetically engineered products made by Monsanto that have the glyphosate-tolerance trait, including DeKalb branded corn, are sold under the trademark Roundup Ready® in Delaware and elsewhere in the United States.

D. Syngenta Has a Reasonable Apprehension of Being Sued by DeKalb for Infringement of the Lundquist '587 and '877 Patents

1. DeKalb and Its Parent Monsanto Have Already Filed Three Patent Infringement Lawsuits Against Syngenta Since May 2004 Relating to GA21 Corn

29. Within the past two-and-a-half years DeKalb and Monsanto have sued Syngenta three separate times for patent infringement, in three separate courts, on four separate patents, all relating to the same allegedly infringing Syngenta product: glyphosate-tolerant GA21 corn.

30. The first in the series of GA21 lawsuits was brought in this Court by Monsanto on May 12, 2004, the same day that Syngenta publicly announced its acquisition of rights to GA21 corn from Bayer CropScience. Monsanto alleged that Syngenta's proposed making and using of GA21 corn infringed U.S. Patent No. 4,940,835 ("the Shah patent"), entitled "Glyphosate-Resistant Plants." *Monsanto Co. v. Syngenta Seeds, Inc.*, No. 04-305-SLR (D. Del. filed May 12, 2004).

31. Shortly thereafter, on July 27, 2004, DeKalb sued Syngenta in the Northern District of Illinois, alleging that Syngenta had infringed U.S. Patent Nos. 5,538,880 ("the Lundquist '880 patent"), entitled "Method for Preparing Fertile Transgenic Corn Plants," and 6,013,863 ("the Lundquist '863 patent"), entitled "Fertile Transgenic Corn Plants." *DeKalb Corp. v. Syngenta Seeds, Inc.*, No. 04-CV-50323 (N.D. Ill. filed July 27, 2004) ("the Lundquist action"). DeKalb alleged that Syngenta's proposed making and using of GA21 corn infringed the Lundquist '880 and '863 patents.

32. The Lundquist '880 and '863 patents purport to claim processes for producing herbicide- or glyphosate-resistant transgenic corn, respectively. DeKalb's Illinois complaint asserted that Syngenta infringed the Lundquist patents "by at least making and using corn containing genes that confer resistance to the herbicide glyphosate." Thus, in both the Shah and Lundquist actions, Monsanto and DeKalb asserted that Syngenta had infringed claims relating to making and using GA21 corn containing a gene that confers resistance to the herbicide glyphosate.

33. On May 19, 2005, the Illinois district court granted Syngenta's motion to transfer the Lundquist action to this Court in major part because GA21 corn products were accused in both actions and the subject matter of the Lundquist and Shah patents was closely related. On

March 24, 2005, this Court consolidated the Shah action with a pending antitrust case brought by Syngenta against Monsanto also relating to Syngenta's efforts to market GA21 corn products. In that antitrust action, Syngenta alleges, *inter alia*, that Monsanto and DeKalb's Shah and Lundquist actions were objectively baseless. *Syngenta Seeds, Inc. v. Monsanto Co.*, C.A. No. 04-CV-908 SLR (D. Del. filed July 28, 2004). Monsanto asserted state law counterclaims (which remain pending) in the antitrust case relating to Syngenta's GA21 corn products and acquisition of Advanta. Subsequently, this Court consolidated the Shah and Lundquist actions on August 23, 2005.

34. On May 10, 2006, this Court granted summary judgment in favor of Syngenta, finding that Syngenta had not infringed the Lundquist '880 and '863 patents and that the Shah patent was invalid for failure to meet the enablement requirement of 35 U.S.C. § 112.

35. Immediately after this Court's decision in favor of Syngenta, Monsanto publicly announced its intention to appeal the Shah and Lundquist decisions to the United States Court of Appeals for the Federal Circuit. Subsequently, Monsanto filed its notice of appeal, and the appeal is currently pending. *Monsanto Co. v. Syngenta Seeds, Inc.*, Appeal No. 06-1472 (Fed. Cir.).

36. Having lost its patent infringement case against Syngenta in this Court, on August 11, 2006, DeKalb sued Syngenta and its affiliates in a different court, the Eastern District of Missouri, alleging infringement of U.S. Patent No. 5,554,798 ("the Lundquist '798 patent"), entitled "Fertile Glyphosate-Resistant Transgenic Corn Plants." *DeKalb Genetics Corp. v. Syngenta Seeds, Inc.*, No. 4 06CV01191MLM (E.D. Mo. filed Aug. 11, 2006).

37. DeKalb's Missouri complaint mirrors its complaint in the prior case in this Court and alleges that Syngenta has "infringed and continue[s] to infringe one or more claims of the

'798 patent by at least making corn containing genes that confer resistance to the herbicide glyphosate." Again, DeKalb is alleging that Syngenta's GA21 corn products infringe one of its Lundquist patents.

38. The Lundquist '798 patent purports to claim a fertile transgenic corn plant containing heterologous DNA that confers resistance to the herbicide glyphosate.

39. The Lundquist '798 patent is in the same patent family as the Lundquist '880 and '863 patents that were previously before this Court in the Lundquist action. The specification of the Lundquist '798 patent is similar to the specifications of the Lundquist '880 and '863 patents. The same alleged inventors (Ronald Lundquist and David Walters) are named on the Lundquist '880, '863, and '798 patents. True and correct copies of the Lundquist '880, '863, and '798 patents are attached as Exhibits C, D, and E, respectively.

2. The Lundquist '587 Patent Is Closely Related to the Lundquist '880 and '863 Patents That DeKalb Has Already Asserted Against Syngenta

40. On information and belief, the Lundquist '587 patent, entitled "Method for Preparing Fertile Transgenic Corn Plants," was granted to DeKalb by the United States Patent and Trademark Office ("PTO") on September 20, 2005. Exh. A.

41. On information and belief, since the date of issuance of the Lundquist '587 patent, DeKalb has been the owner of the entire right, title, and interest in the patent.

42. The Lundquist '587 patent purports to claim a method of producing fertile transgenic *Zea mays* (corn) plants via the same microprojectile bombardment process that is the subject of the Lundquist '880 and '863 patents that were adjudicated by this Court in the Lundquist action.

43. The Lundquist '587 patent is in the same patent family as the Lundquist '880 and '863 patents that were before this Court in the Lundquist action. The specification of the

Lundquist '587 patent is similar to the specifications of the Lundquist '880 and '863 patents previously asserted by DeKalb against Syngenta. The same alleged inventors (Ronald Lundquist and David Walters) are named on the Lundquist '587, '880, and '863 patents.

44. The claims of the Lundquist '587 patent are closely related to the claims of the Lundquist '880 and '863 patents, which this Court previously determined had not been infringed by Syngenta. For example, claim 1 of the Lundquist '587 patent purports to claim:

> A method for producing a fertile transgenic *Zea mays* plant, comprising the steps of: (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, wherein said DNA comprises a selectable marker gene; (ii) selecting a population of transformed *Zea mays* cells; and (iii) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is heritable, to yield transgenic progeny *Zea mays* plants.

Similarly, claim 1 of the Lundquist '863 patent, previously asserted against Syngenta, purports to claim:

> A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, wherein said DNA comprises at least a screenable marker gene; (ii) selecting a population of transformed cells expressing the selectable marker gene; and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is expressed so as to impart glyphosate resistance to said transgenic plant and is transmitted through a normal sexual cycle of said transgenic plant to progeny plants.

Likewise, claim 1 of the Lundquist '880 patent, also previously asserted against Syngenta, purports to claim:

> A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, (ii) identifying or selecting a population of transformed cells, and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, and imparts herbicide resistance thereto.

3. The Lundquist '877 Patent Is Also Closely Related to the Lundquist '880 and '863 Patents That DeKalb Has Already Asserted Against Syngenta

45. On information and belief, the Lundquist '877 patent, entitled "Method for Preparing Fertile Transgenic Corn Plants," was granted to DeKalb by the PTO on July 23, 1996. Exh. B.

46. On information and belief, since the date of issuance of the Lundquist '877 patent, DeKalb has been the owner of the entire right, title, and interest in the patent.

47. The Lundquist '877 patent purports to claim a process of producing fertile transgenic *Zea mays* (corn) plants via the same microprojectile bombardment process that is the subject of the Lundquist '880 and '863 patents that were previously adjudicated by this Court in the Lundquist action.

48. The Lundquist '877 patent is in the same patent family as the Lundquist '880 and '863 patents that were previously before this Court in the Lundquist action. The specification of the Lundquist '877 patent is similar to the specifications of the Lundquist '880 and '863 patents previously asserted by DeKalb against Syngenta. The same alleged inventors (Ronald Lundquist and David Walters) are named on the Lundquist '877, '880, and '863 patents.

49. The claims of the Lundquist '877 patent are closely related to the claims of the Lundquist '880 and '863 patents, which this Court previously determined had not been infringed by Syngenta. For example, claim 1 of the Lundquist '877 patent purports to claim:

> A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) establishing a regenerable embryogenic callus culture from a *Zea mays* plant to be transformed, (ii) transforming said culture by bombarding it with DNA-coated microprojectiles, (iii) identifying or selecting a transformed cell line, and (iv) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny and imparts herbicide or insect resistance thereto.

Similarly, claim 1 of the Lundquist '863 patent, previously asserted against Syngenta, purports to claim:

> A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, wherein said DNA comprises at least a screenable marker gene; (ii) selecting a population of transformed cells expressing the selectable marker gene; and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is expressed so as to impart glyphosate resistance to said transgenic plant and is transmitted through a normal sexual cycle of said transgenic plant to progeny plants.

Likewise, claim 1 of the Lundquist '880 patent, also previously asserted against Syngenta, purports to claim:

> A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, (ii) identifying or selecting a population of transformed cells, and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, and imparts herbicide resistance thereto.

50. The Lundquist '877 and '587 patents are also related and share similar specifications and the same named inventors. The Lundquist '587 patent is a continuation of U.S. Patent Application Serial No. 07/974,379, which was the patent application that matured into the Lundquist '877 patent.

4. DeKalb Continues to Prosecute Patent Applications in the Lundquist Patent Family in an Effort to Secure Additional Patent Claims to Assert Against Syngenta's GA21 Corn Products

51. On information and belief, DeKalb is continuing to prosecute patent applications directed to glyphosate-resistant corn in an effort to secure additional patent claims to assert against Syngenta's GA21 corn products.

52. DeKalb has filed approximately 32 related patent applications with the PTO in the name of Lundquist and Walters, including a new patent application filed as recently as August 9,

2006. The Lundquist '880, '863, '587, and '877 patents are among the related patents in this family of related patents and applications ("the Lundquist patent family").

53. Despite the fact that the alleged inventors, Lundquist and Walters, never worked with glyphosate and never created any glyphosate-resistant corn product, DeKalb continues to pursue patent claims in the Lundquist patent family in an attempt to secure further claims covering glyphosate-resistant corn. Lundquist and Walters did not invent glyphosate-resistant corn or describe that invention in their patent applications in the Lundquist patent family. All patent claims in the Lundquist patent family purporting to cover that subject matter, which Lundquist and Walters did not invent, are invalid.

54. One pending application in the Lundquist patent family is U.S. Patent Application Serial No. 10/919,228 ("the Lundquist '228 application"). The Lundquist '228 application purports to claim a method of rendering corn plants resistant to the herbicide glyphosate.

55. On July 18, 2006, the PTO issued a Notice of Allowance in the Lundquist '228 application. On September 18, 2006, DeKalb paid the required issue fee associated with the Lundquist '228 application. The Lundquist '228 application is therefore ready for issuance by the PTO, which could occur any time now and is expected soon.

56. On information and belief, DeKalb intends to assert the patent issuing from the Lundquist '228 application against Syngenta upon its issuance. The allowed claims of the Lundquist '228 application, when issued as patent claims, will not be infringed by Syngenta and/or will be invalid. Syngenta reserves the right, upon issuance of the Lundquist '228 application as a patent, to amend this Complaint to seek a declaratory judgment that the patent claims issuing on the Lundquist '228 application are not infringed by Syngenta and/or are invalid.

57. In sum, Syngenta has a reasonable apprehension of being sued by DeKalb for alleged infringement of the Lundquist '587 and '877 patents (and any patent issuing on the Lundquist '228 application) in view of the fact that (1) within the past two-and-a-half years DeKalb and Monsanto have already sued Syngenta three separate times for patent infringement, in three separate courts, on four separate patents, all with respect to Syngenta's GA21 corn products; (2) the Lundquist '587 and '877 patents disclose and claim similar subject matter as the closely related Lundquist '880 and '863 patents that DeKalb previously asserted against Syngenta in this Court; and (3) DeKalb continues to prosecute patent applications in the Lundquist patent family, including the Lundquist '228 application, which will soon issue as a patent, in an effort to secure additional patent claims to assert against Syngenta's GA21 corn products.

COUNTS

First Count: Declaratory Judgment of Non-Infringement of U.S. Patent No. 6,946,587

58. Syngenta hereby restates and realleges the allegations set forth in paragraphs 1 through 57 of this complaint and incorporates them by reference.

59. Syngenta has not infringed and is not infringing, literally or under the doctrine of equivalents, any valid and enforceable claim of the Lundquist '587 patent, directly, contributorily, or by inducement.

Second Count: Declaratory Judgment of Invalidity of U.S. Patent No. 6,946,587

60. Syngenta hereby restates and realleges the allegations set forth in paragraphs 1 through 59 of this complaint and incorporates them by reference.

61. Syngenta cannot be liable for infringement of the Lundquist '587 patent because the claims are invalid for failure to satisfy one or more of the statutory requirements of the Patent Laws of the United States, including 35 U.S.C. §§ 102, 103, and/or 112.

Third Count: Declaratory Judgment of Non-Infringement of U.S. Patent No. 5,538,877

62. Syngenta hereby restates and realleges the allegations set forth in paragraphs 1 through 61 of this complaint and incorporates them by reference.

63. Syngenta has not infringed and is not infringing, literally or under the doctrine of equivalents, any valid and enforceable claim of the Lundquist '877 patent, directly, contributorily, or by inducement.

Fourth Count: Declaratory Judgment of Invalidity of U.S. Patent No. 5,538,877

64. Syngenta hereby restates and realleges the allegations set forth in paragraphs 1 through 63 of this complaint and incorporates them by reference.

65. Syngenta cannot be liable for infringement of the Lundquist '877 patent because the claims are invalid for failure to satisfy one or more of the statutory requirements of the Patent Laws of the United States, including 35 U.S.C. §§ 102, 103, and/or 112.

PRAYER FOR RELIEF

WHEREFORE, Syngenta respectfully requests that this Court:

1. Declare that Syngenta has not infringed any claim of the Lundquist '587 or '877 patents;

2. Declare that each claim of the Lundquist '587 and '877 patents is invalid;

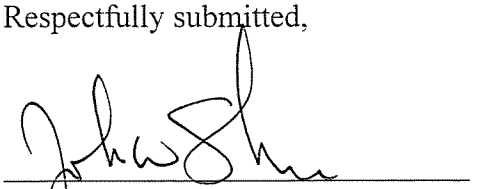
3. Permanently enjoin DeKalb, its officers, agents, servants, employees, and attorneys, and those other persons or entities in active concert or participation with it, from

asserting (either in a lawsuit or in any other manner) that Syngenta has infringed any claim of the Lundquist '587 or '877 patents;

4. Declare that this case is exceptional under 35 U.S.C. § 285 and award Syngenta its costs, disbursements, and reasonable attorney fees in connection with this action; and

5. Award Syngenta such other and further relief as this Court may deem just and equitable.

Respectfully submitted,



John W. Shaw (#3362)
YOUNG CONAWAY STARGATT & TAYLOR, LLP
The Brandywine Building
1000 West Street
P.O. Box 391
Wilmington, DE 19899-0391
(302) 571-6600
jshaw@ycst.com

Of counsel:

Michael J. Flibbert
Howard W. Levine
Sanya Sukduang
Jennifer A. Johnson
FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.
901 New York Avenue, N.W.
Washington, DC 20001-4413
(202) 408-4000

Attorneys for Plaintiffs

Dated: October 5, 2006

EXHIBIT A

US006946587B1

(12) **United States Patent**
Lundquist et al.

(10) **Patent No.: US 6,946,587 B1**
(45) **Date of Patent: *Sep. 20, 2005**

(54) **METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS**

(75) Inventors: **Ronald C. Lundquist**, Minnetonka, MN (US); **David A. Walters**, Bloomington, MN (US)

(73) Assignee: **DeKalb Genetics Corporation**, DeKalb, IL (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **08/677,695**

(22) Filed: **Jul. 10, 1996**

Related U.S. Application Data

(63) Continuation of application No. 07/974,379, filed on Nov. 10, 1992, now Pat. No. 5,538,877, which is a continuation of application No. 07/467,983, filed on Jan. 22, 1990, now abandoned.

(51) **Int. Cl.**[7] **C12N 15/82**; C12N 15/31; C12N 15/32; C12N 15/54; A01H 5/00

(52) **U.S. Cl.** **800/293**; 800/275; 800/288; 800/300.1; 800/302; 800/320.1; 435/194; 435/424; 435/430; 435/430.1; 435/470; 536/23.7; 536/23.71

(58) **Field of Search** ..... 800/275, 288, 800/300.1, 302, 320.1, 293, 205; 435/194, 424, 430, 430.1, 470, 172.3, 172.1; 536/23.7, 23.71; 41/55, DIG. 1

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,370,160	A	1/1983	Ziemelis	71/117
4,399,216	A	8/1983	Axel et al.	435/6
4,520,113	A	5/1985	Gallo et al.	436/504
4,535,060	A	8/1985	Comai	435/172
4,536,475	A	8/1985	Anderson	435/172.3
4,559,301	A	12/1985	Turner	435/76
4,559,302	A	12/1985	Ingolia	435/76
4,581,847	A	4/1986	Hibberd et al.	47/58
4,634,665	A	1/1987	Axel et al.	435/68
4,642,411	A	2/1987	Hibberd et al.	800/1
4,665,030	A	5/1987	Close	
4,666,844	A	5/1987	Cheng	435/240
4,683,202	A	7/1987	Mullis	435/91
4,708,818	A	11/1987	Montagnier et al.	435/5
4,727,028	A	2/1988	Santerre et al.	435/240
4,743,548	A	5/1988	Crossway et al.	435/172
4,761,373	A	8/1988	Anderson et al.	435/172.3
4,806,483	A	2/1989	Wang	435/240
4,885,357	A	12/1989	Larkins et al.	530/373
4,886,878	A	12/1989	Larkins et al.	536/26
4,940,835	A	7/1990	Shah et al.	800/205
4,945,050	A	7/1990	Sanford et al.	435/172.1
4,956,282	A	9/1990	Goodman et al.	435/69.51
4,971,908	A	11/1990	Kishore et al.	435/172
5,001,060	A	3/1991	Peacock et al.	435/172
5,004,863	A	4/1991	Umbeck	800/205
5,013,658	A	5/1991	Dooner et al.	435/172.3
5,015,580	A	5/1991	Christou et al.	435/172
5,034,322	A	7/1991	Rogers et al.	435/172
5,036,006	A	7/1991	Sanford et al.	435/170.1
5,049,500	A	9/1991	Arnizen et al.	435/172
5,077,399	A	12/1991	Brauer et al.	536/27
5,082,767	A	1/1992	Hatfield et al.	435/6
5,094,945	A	3/1992	Comai	435/172
5,097,093	A	3/1992	Vandeventer et al.	800/200
5,110,732	A	5/1992	Benfey et al.	435/172
5,134,074	A	7/1992	Gordon et al.	435/240
5,145,777	A	9/1992	Goodman et al.	435/172.3
5,164,310	A	11/1992	Smith et al.	435/172.3
5,177,010	A	1/1993	Goldman et al.	435/172
5,187,073	A	2/1993	Goldman et al.	435/172
5,188,642	A	2/1993	Shah et al.	47/58
5,188,958	A	2/1993	Moloney et al.	435/240
5,196,342	A	3/1993	Donovan	435/320.1
5,215,912	A	6/1993	Hoffman	435/240.4
5,231,020	A	7/1993	Jorgensen et al.	435/172.3
5,240,841	A	8/1993	Johnston et al.	435/172.3
5,250,515	A	10/1993	Fuchs et al.	514/12
5,254,799	A	10/1993	DeGrave et al.	800/205
5,258,300	A	11/1993	Glassman et al.	435/240
5,268,463	A	12/1993	Jefferson	536/23
5,273,894	A	12/1993	Strauch et al.	435/129
5,276,268	A	1/1994	Strauch et al.	800/205
5,278,325	A	1/1994	Strop et al.	554/12
5,290,924	A	3/1994	Last et al.	536/241
5,310,667	A	5/1994	Eichholtz et al.	435/172.3

(Continued)

FOREIGN PATENT DOCUMENTS

AU	80893/87	12/1988	C12N/15/87
CA	2032443 A1	12/1990	C12N/15/87
DE	3738874 A1	11/1988	
DE	3 738 874 A1	11/1988	A01H/1/06
EP	126537 A2	4/1983	

(Continued)

OTHER PUBLICATIONS

Vasil et al. Theoretical and Applied Genetics 73: 793–798 (1987).*

Andrews, D.L., et al., "Characterization of the Lipid Acyl Hydrolase Activity of the Major Potato (*Solanum tuberosum*) Tuber Protein, Patatin, by Cloning and Abundant Expression in a Baculovirus Vector", *Biochem. J., 252,* 199–206 (1988).*

(Continued)

Primary Examiner—David T. Fox
(74) *Attorney, Agent, or Firm*—Fulbright & Jaworski L.L.P.

(57) **ABSTRACT**

Fertile transgenic *Zea mays* (corn) plants which stably express heterologous DNA which is heritable are disclosed along with a process for producing said plants. The process comprises the microjectile bombardment of friable embryogenic callus from the plant to be transformed. The process may be applicable to other graminceous cereal plants which have not proven stably transformable by other techniques.

19 Claims, 10 Drawing Sheets

U.S. PATENT DOCUMENTS

5,350,689	A		9/1994	Shillito et al.	435/240
5,352,605	A		10/1994	Fraley et al.	435/240
5,371,003	A		12/1994	Murry et al.	435/172
5,371,015	A		12/1994	Sanford et al.	435/287
5,380,831	A	*	1/1995	Adang et al.	536/23.71
5,436,393	A		7/1995	Rocha-Sosa et al.	800/205
5,464,763	A		11/1995	Schilperoort et al.	435/172.3
5,484,956	A		1/1996	Lundquist et al.	800/205
5,489,520	A		2/1996	Adams et al.	
5,495,071	A		2/1996	Fischhoff et al.	800/205
5,500,365	A		3/1996	Fischhoff et al.	435/240.4
5,508,468	A		4/1996	Lundquist et al.	800/205
5,538,877	A		7/1996	Lundquist et al.	435/172.3
5,538,880	A		7/1996	Lundquist et al.	435/172.3
5,550,318	A		8/1996	Adams et al.	
5,554,798	A		9/1996	Lundquist et al.	800/205
5,561,236	A		10/1996	Leemans et al.	
5,565,347	A	*	10/1996	Fillatti et al.	435/172.3
5,567,600	A		10/1996	Adang et al.	
5,567,862	A		10/1996	Adang et al.	
5,576,203	A		11/1996	Hoffman	435/172.3
5,578,702	A		11/1996	Adang	
5,580,716	A		12/1996	Johnston et al.	435/5
5,589,616	A		12/1996	Hoffman	
5,595,733	A		1/1997	Carswell et al.	424/93.21
5,596,131	A		1/1997	Horn et al.	800/205
5,623,067	A		4/1997	Vanderkerckhove et al.	536/24.1
5,641,876	A		6/1997	McElroy et al.	536/24.1
5,668,298	A	*	9/1997	Waldron	800/288
5,677,474	A		10/1997	Rogers	800/205
5,693,507	A		12/1997	Daniell et al.	435/172.3
5,780,708	A		7/1998	Lundquist et al.	800/205
5,886,244	A		3/1999	Tomes et al.	800/293
5,990,387	A		11/1999	Tomes et al.	800/293
5,990,390	A		11/1999	Lundquist et al.	800/302
6,013,863	A		1/2000	Lundquist et al.	800/293
6,020,539	A		2/2000	Goldman et al.	800/294
6,025,545	A		2/2000	Lundquist et al.	800/293
6,258,999	B1		7/2001	Tomes et al.	800/300.1

FOREIGN PATENT DOCUMENTS

EP	131623	B1	1/1984	
EP	141373	A3	5/1985	
EP	0 142 924	A2	5/1985	C12N/15/00
EP	154204	A2	9/1985	
EP	160390	A2	11/1985	
EP	193259	A1	9/1986	
EP	204549	A2	10/1986	
EP	202668	A2	11/1986	
EP	242236	A1	10/1987	
EP	242246	A1	11/1987	
EP	299552	A1	1/1988	
EP	257472	A2	3/1988	
EP	262971	A2	5/1988	
EP	0 269 601	A2	6/1988	C12N/15/00
EP	270356	A2	6/1988	
EP	271408		6/1988	
EP	275069	A2	7/1988	
EP	280400	A2	8/1988	
EP	282164	A2	9/1988	
EP	289479	A2	11/1988	
EP	290395	A2	11/1988	
EP	292435	A1	11/1988	
EP	301749	A2	2/1989	
EP	353908	A2	7/1989	
EP	0 331 083	A2	9/1989	C12N/15/00
EP	331855	A2	9/1989	
EP	334539	A2	9/1989	
EP	0 335 528	A2	10/1989	C12N/15/00
EP	348348	A2	12/1989	
EP	0 348 348	A2	12/1989	A01N/65/00
EP	442174	A1	4/1991	
GB	2159173	A	11/1985	
JP	61-134343		5/1984	
NL	8801444		1/1990	
WO	85/01856		5/1985	
WO	85/02972		7/1985	
WO	85/02973		7/1985	A01J/7/00
WO	86/01536		3/1986	C12P/15/00
WO	86/03776		7/1986	C12N/15/00
WO	87/04181		7/1987	C12N/1/00
WO	87/05629		9/1987	
WO	88/08034		10/1988	C12P/21/00
WO	89/04371		5/1989	C12N/21/00
WO	89/10396		11/1989	
WO	89/11789		12/1989	A01H/1/00
WO	89/12102		12/1989	
WO	90/01869		3/1990	A01H/1/00
WO	90/02801		3/1990	C12N/15/32
WO	90/10691		8/1990	

OTHER PUBLICATIONS

Fromm, M.E., et al., "Inheritance and Expression of Chimeric Genes in the Progeny of Transgenic Maize Plants", *Bio/Technology, 8,* 833–839 (1990).*

Jaynes, J.M., et al., "Plant Protein Improvement by Genetic Engineering: Use of Synthetic Genes", *Trends in Biotechnology, 4,* 314–320 (Dec. 1986).*

Messing, J., "Corn Storage Protein: A Molecular Genetic Model", *Division of Energy Biosciences–Summaries of FY 1990 Activities,* p. 70, Abstract No. 135 (1990).*

"Bullets Transforms Plant Cells", *Agricell Report, 9,* 5, (Jul. 1987).*

"Catalog, Handbook of Fine Chemicals", *Aldrich Chem. Co.,* p. 508 (1988).*

"Cornell U. Gene Gun Hits Biotech Bullseye", *Agriculture Technology,* p. 13.*

"Dalapon," In: Merck Index, 11th Edition, S. Budavae, (ed), Merck and Co., pp. 405–406 (1989).*

"Dalapon," In: Merck Index, 11th Edition, S. Budavae, (ed), Merck and Co., pp. 405–406 (1989).*

EPO Notice Regarding Publication of Biobliographic Data for EPO 0485506.*

"Herbicide–Resistant Corn", *CT Academy of Science and Engineering, Case Reports, 5,* 6 (1990).*

International Search Report, PCT/US 90/09699, Mailed Aug. 16, 1995.*

International Search Report, PCT/US 90/04462, Mailed Jan. 15, 1991.*

Patent Family Record for Australian Patent 87 80 893.*

"Shotgunning DNA into Cells", *Genetic Engineering News,* (Jul.–Aug. 1987).*

M. J. Adang, et al., "Characterized Full–Length and Truncated Plasmid Clones of the Crystal Protein of *Bacillus thuringiensis* subsp. *kurstaki* HD–73 and Their Toxicity to *Manduca sexta", Gene, 36,* 289–300 (1985).*

H. Ahokas, "Transfection of Germinating Barley Seed Electrophorectically with Exogenous DNA", *Theor. Appl. Genet., 77,* 469–472 (1989).*

H. Ahokes, "Electrophorectic transfection of cereal grain with exogenous nucleic acid", *Soc., biochem Biophys. Microbio. Fen., Biotieteen Paivat* (*Bioscience Days*), Abstracts, Technical University of Helsinki, Espoo, p. 2 (1989).*

S. B. Altenbach, et al., "Cloning and Sequence Analysis of a cDNA Encoding a Brazil Nut Protein Exceptionally Rich in Methionine", *Plant Mol. Biol. 8,* 239–250 (1987).*

S. B. Altenbach, et al., "Enhancement of the Methionine Content of Seed Proteins by the Expression of a Chimeric Gene Encoding a Methionine–Rich Protein in Transgenic Plants", *Plant. Mol. Biol., 13,* 513–522 (1989).*

C. Ampe, et al., "The Amino–Acid Sequence of the 2S Sulphur–Rich Storage Protein Accumulation in Maize", *Theor. Appl. Genet., 78,* 761–767 (1989).*

C. L. Armstrong, et al., "Establishment and Maintenance of Friable, Embryogenic Maize Callus and the Involvement of L–Proline", *Planta, 164,* 207–214 (1985).*

C. Armstrong, et al., "Genetic and Cytogenetic Variation in Plants Regenerated from Organogenic and Friable Embryogenic Tissue Cultures in Maize", *Biol. Abstracts, 85,* Abstract 117662 (1988).

R. F. Barker, et al., "Nucelotide Sequence of the T–DNA Region from the *Agrobacterium tumefaciens* Octopone Ti Plasmid pTi5955", *Plant Mol. Biol., 2,* 335–350 (1983).

M. S. Benner, et al., "Genetic Analysis of Methionine–Rich Storage Protein Accumulation in Maize", *Theor. Appl. Genet., 78,* 761–767 (1989).

M. Bevan, et al., "A Chimaeric Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation", *Nature, 304,* 184–187 (1983).

M. Bevan, et al., "Structure and Transcription of the Nopaline Synthase Gene Region of T–DNA", *Nuc. Acids Res., 11,* 369–385 (1983).

A. N. Binns, "Agrobacterium–mediated gene delivery and the biology of host range limitations", *Physiologia Plantarum, 79,* 135–139 (1990).

G. Booy, et al., "Attempted Pollen–Mediated Transformation of Maize", *J. Plant Physiol., 135,* 319–324 (1989).

M. I. Boulton, et al., "Specificity of Agrobacterium–mediated delivery of maize stread virus DNA to members of the Gramineae", *Plant Molecular Biology, 12,* 31–40 (1989).

J S Boyer, "Water Deficits and Photosynthesis", *In: Water Deficits and Plant Growth,* vol. IV, Kozlowski, T.T., (ed), Academic Press, New York, pp. 153–190 (1976).

W. J. Brill, "Agricultural Microbiology", *Scientific American, 245,* 199–215 (Sep. 1981).

V. Buchanan–Wollaston, et al., "Detoxification of the Herbicide Dalapon by Transformed Plants", *J. of Cell. Biochem, 13D,* Abstract No. M503 (1989).

J. Callis, et al., "Introns Increase Gene Expression in Cultures Maize Cells", *Genes and Development, 1,* 1182–1200 (1987).

J. Cao, et al., "Transformation of Rice and Maize Using the Biolistic Process", In: *Plant Gene Transfer,* Alan R. Liss, Inc., pp. 21–33 (1990).

N. C. Carpita, "The Biochemistry of Growing Cell Walls", In: *Physiology of Cell Expansion During Plant Growth,* Cosgrove, D. J. et al, (eds) Am. Soc. Plant Physiol, pp. 28–100 (1987).

V. Chandler, et al., "Two Regulatory Genes of the Maize Anthocyanin Pathway Are Homologous: Isolation of B Utilizing R Genomic Sequences", *The Plant Cell, i,* 1175–1183 (1989).

P. J. Charest, et al., "Factors Affecting the Use of Chloramphenicol Acetyltransferase as a Marker for Brassica Genetic Transformation", *Plant Cell Reports, 7,* 628–631 (1989).

P. S. Chourey, et al., "Callus Formation from Protoplasts of a Maize Cell Culture", *Theor. Appl. Genet. 59,* 341–344 (1981).

Christou, et al., "Cotransformation Frequencies of Foreign Genes in Soybean Cell Cultures", *Theor. Appl. Genet. 79,* 337–341 (1990).

P. Christou, et al., "Opine Synthesis in Wild Type Plant Tissue", *Plant Physiol. 82,* 218–221 (1986).

P. Christou, et al., "Soybean Genetic Engineering–Commercial Production of Transgenic Plants", *Trends Biotechnol. 8,* 145–151 (1990).

P. Christou, et al., "Stable Transformation of Soybean Callus DNA–Coated Gold Particles", *Plant Physiol. 87,* 671–674 (1988).

C. C. Chu, et al., "Establishment of an Efficient Medium for Another Culture of Rice Through Comparative Experiments on the Nitrogen Sources", *Sci. Sein. (Peking),* 13, 659–668 (1975).

F. Cocking, et al., "Gene Transfer in Cereals", *Science, 236,* 1259–1262 (1987).

Coe, et al., "The Genetics of Corn", In: *Corn and Corn Improvement,* 2nd Edition, Sprague, G.F. (ed.), American Soc. Agronomy, Inc., Madison, WI, p. 138 (1977).

L. Comai, et al., "Expression in Plants of a Mutant aroA Gene from *Salmonella typhimurium* Confers Tolerance to Glyphosate", *Nature, 317,* 741–744, (Oct. 1985).

Creissen, et al., "Agrobacterium—and microprojectile—Mediated Viral DNA Delivery into Barley Microspore–Derived Cutures", *Plant Cell Reports, 8,* 680–863 (Apr. 1990).

A. Crossway, et al., "Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts", *Mol. Gen. Genet., 202,* 179–185 (1986).

A. Darvill, et al., "The Primary Cell Walls of Flowering Plants", In: *The Biochemistry of Plants, vol. 1,* pp. 91–162 (1980).

B. Dauce–LeReverand, et al., "Improvement of *Escherichia coli* Strains Overproducing Lysine Using Recombinant DNA Techniquest", *Eur. J. Appl. Microbiol. Biotechnol., 15,* 227–231 (1982).

M. De Block, et al., "Engineering herbicide resistance on plants by expression of a detoxifying enzyme", *EMBO J., 6,* 2513–2518 (1987).

W. De Greef, et al., "Evaluation of herbicide resistance in transgenic crops under field conditions", *Bio/Technol, 7,* 61–64 (1989).

R. Dekeyser, et al., "Evaluation of Selectable Markers for Rice Transformation", *Plant Physiol, 90,* 217–223 (1989).

R. A. Dekeyser, et al., "Transient Gene Expression in Intact and Organized Rice Tissues", *The Plant Cell, 2,* 591–602 (1990).

Dewald, et al., "Plant regeneration from inbred maize suspensions", *VIIth International Congress on Plant Tissue and Cell Culture,* p. 12, Abstract No. A1–36 (Jun. 24–29, 1990).

J. R. Dewet, et al., "Cloning of Firefly Luciferase cDNA and the Expression of active Luicferase in *Escherichia coli", Pro. Nat. Acad. Sci. USA, 82,* 7870–7873 (1985).

J.m. J. Dewet, et al., "Exogenous gene transfer in maize (*Zea mays*) using DNA–treated pollen", In: *The experimental manipulation of ovule tissues,* Chapman, G.P., et al(eds), Longman, New York, pp. 197–209 (1985).

D. A. Evans, et al., "Somaclonal Variation–Genetic Basis and Breeding Applications", *Trends Genet., 5,* 46–50 (1989).

P. Fransz, et al., "Cytodifferentiation during callus initation and somatic embryogenesis in *Zea mays* L", Ph.D. thesis, U of Wageningen Press, The Netherlands (1988).

J. C. Freeling, et al., "Development Potentials of Maize Tissue Cultures", *Maydica, XXL,* 97–112 (Jul., 1977).

Freiberg, "More Researchers Discover Corn Transformation Technology", *AG Biotechnology News,* p. 26 (1990).

M. Fromm, et al., "Expression of Genes Transfected into Monocot and Dicot Plant Cells by Electroportion", *Proc. Nat. Acad. Sci. USA, 82,* 6824–5828 (1985).

M. E. Fromm, et al., "Stable Transformation of Maize after Gene Transfer by Electroporation", *Nature, 319,* 791–793 (1986).

S. C. Fry, "Introduction to the Growing Cell Wall", In: *The Growing Plant Cell Wall: Chemical and Metabolic Analysis,* Longman Scientific and Technical, New York, pp. 1–5, 102–109 (1988).

M. Geiser, et al., "The Hypervariable Region on the Genes Coding for Entomopahtogenic Crystal Proteins of *Bacillus thuringiensis:* Nucleotide Sequence of the kurhd1 gene for subsp. *kurstaki* HD1", *Gene, 48,* 109–108 (1986).

S. A. Goff, et al., "Transactivation of Anthocyanin Biosynethic Genes Following Transfer of B Regulatory Genes into Maize Tissues", *EMBO J., 9,* 2517–2522 (1990).

W. J. Gordon–kamm, et al., "Stable Transformation of Embryonic Maize Cultures by Microprojectile Bombardment", *J. Cellular Biochem., 13D,* p. 259, Abstract No. M122 (1989).

O. Gould, et al., "Shoot Tip Culture as a Potential Transformation System", Abstracts, *Beltwide Cotton production research conferences,* New Orleans, LA, p. 91 (1988).

A. Graves, et al., "The transformation of *Zea mays* seedlings with *Agrobacterium tumefacians", Plant Mol. Biol., 7,* 43–50 (1986).

C. Green, et al., "Plant Regeneration in Tissue Cultures of Maize", *Crop. Sci., 15,* 417–421 (1975).

C. Green, et al., "Plant Regeneration in Tissue Cultures in Maize", *In: Maize for Biological Research,* Sheridan, W., F., (ed) Plant Mol. Biol. Assoc., pp. 367–372 (1983).

C. Green, et al., "Somatic Cell Genetic System in Corn", *Advances in Gene Technology: Molecular Genetics of Plant and Animals,* Academic Press, Inc., pp. 147–157 (1983).

N. Grimsley, et al., "DNA Transfer from Agrobacterium to *Zea mays* or Brassica by Agroinfection is Dependent on Bacterial Virulence Functions", *Mol. Gen. Genet., 217,* 309–316 (1989).

L. Gritz, et al., "Plasmid–Encoded Hygromycin B Resistance: The Sequence of Hygromycin B Phosphotransferase Gene and Its Expression in *Escherichia coli* and *Saccharomyces cerevisiae", Gene 25,* 179–188 (1983).

F. Guerineau, et al., "Sulfonamide Resistance Gene for Plant Transformation", *Plant Molecular Biology, 15,* 127–136 (1990).

H. Guilley, et al., "Transcription of Cauliflower Mosaic Virus DNA: Detection of Promoter Sequences, and Characterization of Transcripts", *Cell. 30,* 763–773 (Oct. 1982).

A. R. Hallauer, et al., "Corn Breeding", In: *Corn and Corn Improvement,* 3rd edition, Sprague, G.F., et al(eds), Agronomy Soc. Amer., pp. 463–564 (1988).

G. W. Haughn, "Transformation with a Mutant Arabidopsis Acetolactate Synthase Gene Renders Tobacco Resistant to Sulfonylurea Herbicides", *Mol. Gen. Genet., 211,* 266–271 (1988).

R. M. Hauptman, et al., "Evaluation of Selectable Markers for Obtaining Stable Transformants on the Gramineae", *Plant Physil. 86,* 602–606 (1988).

L. Herrera–Estrella, et al., "Use of Reporter Genes to Study Gene Expression in Plant Cells", *In: Plant Molecular Biology Manual B1,* Kluwer Academic Publishers, Dordrecht, pp. 1–22, (1988).

L. M. Hoffman, et al., "A Modified Storage Protein is Synthesized, Processed, and Degraded in the Seeds of Transgenic Plants", *Plant. Mol. Biol., 11,* 717–729 (1988).

L. M. Hoffman, et al., "Synthesis and Protein Body Deposition of Maize 15kD Zein in Transgenic Tobacco Seeds", *EMBO J., 6,* 3213–3221 (1987).

H. Hofte, et al., "Insecticidal Crystal Proteins of *Bacillus thruingiensis", Microbiol. Rev., 53,* 242–255 (1989).

P. J. J. Hooykass, "Transformation of plant cell via Agrobacterium", *Plant Mol. Biol. 13,* 327–336 (1989).

M. Horn, et al., "Transgenic Plants of Orchard Grass(*Dactylis glomerata*L) from Protoplasts", *Chem. Abstracts, 110,* p. 208, Abstract No. 89869a (1989).

M. Horn, et al., "Transgenic Plants of Orchardgras (*Dactylis glomerata*L.) from Protoplasts", *Plant Cell Reports, 7,* 469 (1988).

Y. Huang, et al., "Factors Influencing Stable Transformation of Maize Protoplasts by Electroporation", *Plant Cell, Tissue and Organ Culture, 18,* 281 (1989).

C. Imbrie–milligan, et al., "Microcallus Growth from Maize Protoplasts", *Planta, 171,* 58–64 (1987).

R. Jefferson, "Assaying chimeric genes in plants: the GUS gene fusion system", *Plant Mol. Biol. Rep., 5,* 387–405 (1987).

R. Jefferson, et al., "B–Glucuronidase from *Escherichia coli* as a Gene–Fusion Marker", *Proc. Natl. Acad. Sci. USA, 83,* 8447–8451 (1986).

R. Jefferson, et al., "GUS Fusions: B–Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher Plants", *EMBO J., 6,* 3901–3907 (1987).

M. M. Johri, et al., "Genetic Approaches to Meristem Organization", In: *Maize for Biological Research,* W. F. Sheridan, (ed.), Plant Molecular Biology Association, pp. 301–310 (1982).

H. Jones, et al., "Recent Advances in Plant Electroporation", *Oxford Surveys of Plant Molecular and Cell Biol, 4,* 347–357 (1987).

H. Jones, et al., "Transcient Gene Expression in Electroporated Solanum Protoplasts", *Plant Mol. Biol., 13,* 503–511 (1989).

H. F. Kaeppler, et al., "Silicon Carbide Fiber–Mediated DNA Delivery into Plant Cells", *Plant Cell Rep.,* 9, 415–418 (1990).

K. Kamo, et al., "Establishment and Characterization of Long–Term Embryonic Maize Callus and Cell Suspension Cultures", *Plant Sci., 45,* 111–117 (1986).

K. Kamo, et al., "Regeneration of *Zea mays* L. from Embryogenic Callus", *Bot. Gaz., 146,* 327–334 (1985).

K. N. Kao, et al., "Nutritional Requirements for Growth of *Vicia hajastana* Cells and Protoplasts at a Very Low Population Density in Liquid Media", *Planta, 126,* 105–110 (1978).

K. Kartha, et al., "Transient Expression of Chloramphenicol Acetyl Transferase (CAT) Gene in Barley Cell Cultures and Immature Embryos Through Microprojectile Bombardment", *Plant. Cell. Rep., 8,* 429–432 (1989).

K. Kay, et al., "Duplication of CaMV 35S Promoter Sequences Creates a Strong Enchancer for Plant Genes", *Science, 236,* 1299–1302 (Jun. 5, 1977).

J. Kirihara, et al., "Differential Expression of a Gene for a Methionine–Rich Storage Protein in Maize", *Mol. Gen. Genet., 211,* 477–484 (1988).

J. Kirihara, et al., "Isolation and Sequence of a Gene Encoding a Methionine–Rich 10–kD Zein Protein from Maize", *Gene, 71,* 359–370 (1988).

T. M. Klein, et al., "Factors Influencing Gene Delivery into *Zea mays* Cells by High Velocity Microprojectiles", *Bio/Technol., 6,* 559–563 (1988).

T. Klein, et al., "Genetic Transformation of Maize Cell by Particle Bombardment and the Influence of Mehtylation on Foreign Gene Expression", In: *Gene Manipulation In Plant Improvement II,* Gustafson, J.P., (ed.). Plenum Press, NY, pp. 265–266 (1990).

T Klein, et al., "Genetic Transformation of Maize Cells by Particle Bombardment", *Plant Physiol. 91,* 440–444 (1989).

T. M. Klein, et al., "High–Velocity Microprojectiles for Delivering Nucleic Acids to Living Cells", *Nature, 327,* 70–73 (1987).

T. Klein, et al., "Regulation of Anthocyanin Biosynthetic Genes Introduced into Intact Maize Tissue by Microprojectiles", *Pro. Nat. Acad. Sci., USA, 86,* 6682–6685 (1989).

T. Klein, et al., "Transfer of Foreign Genes into Intact Maize Cells with High–Velocity Microprojectiles", *Pro. Nat. Acad. Sci. USA, 85,* 4305–4309 (1988).

M. Kozak, "Compilation and Analysis of Sequence from the Translational Start Site in Eukaryotic mRNAs", *Nuc. Acids. Res., 12,* 857–871 (1984).

M. Kozak, "Point Mutations Define a Sequence Flanking the AUG Inhitiator Codon that Modulates Translation by Eukaryotic Ribosomes", *Cell, 44,* 283–292 (1986).

C. Kuhlemeier, et al., "Regulation of Gene Expression in Higher Plants", *Ann. Rev. Plant Physiol., 38,* 234–239 (1987).

P. Lazzeri, et al., "In Vitro Genetic Manipulation of Cereals and Grasses", *Ad. Cell Culture, 6,* 291–293 (1988).

J. S. Lee, et al., "Gene Transfer into Inact Cells of Tobacco by Electroporation", *Korean J. Gent., 11,* 65–72 (1989).

J. Levitt, "Growth Regulators", In: *Introduction to Plant Physiology,* The C.V. Mosby Company, St. Louis, p. 241 (1969).

K. Lindsey, et al., "Electroporation of Cells", *Physiologia Plantarum, 79,* 168–172 (1990).

K. Lindsey, et al., "Stable Transformation of Sugarbeet Protoplasts by Electroporation", *Plant Cell. Rep., 8,* 71–74 (1989).

K. Lindsey, et al., "The Permeability of Electroporated Cells and Protoplasts of Sugar Beet", *Planta, 172,* 346–355 (1987).

K. Lindsey, et al., "Transient Gene Expression in Electroporated Protoplasts and Intact Cells of Sugar beet", *Plant Mol. Bio., 10,* 43–52 (1987).

H. Lorz, et al., "Advances in Tissue Cultures and Progress Towards Genetic Transformation of Cereals", *Plant Breeding, 100,* 1–25 (1988).

C. Lu, et al., "Improved Efficiency of Somatic Embryogenesis and Plant Regeneration on Tissue Cultures of Maize(*Zea mays* L__", *Theor. Appl. Genet. 66,* 285–289 (1983).

C. Lu, et al., "Somatic Embryogenesis in *Zea mays* L", *Theor. Appl. Genet. 62,* 109–112 (1982).

S. Ludwig, et al., "A Regulatory Gene as a Novel Visible Marker for Maize Transformation", *Science, 247,* 449–450 (1990).

S. Ludwig, et al., "High Frequency Callus Formation from Maize Protoplasts", *Theor. Appl. Genet., 71,* 344–350 (1985).

S. Ludwig, et al., "Lc, a Member of the Maize R Gene Family Responsible for Tissue–Specific Anthocyanin Production, Encodes a Protein Similar to Transcriptional Activators and Contains the myc–Homology Region", *Proc., Nat. Acad. Sci. USA, 86,* 7092–7096 (1989).

S. Ludwig, et al., "Maize R Gene Family: Tissue Specific Helix Loop Helix Proteins", *Cell, 62,* 849–851 (1990).

H. Lutcke, et al., "Selection of AUG Initiation Codons Differs in Plants and Animals", *EMBO J., 6,* 43–48 (1987).

T. Masumura, et al., "cDNA Cloning of an mRNA Encoding a Sulfur–Rich 10 kDa Prolamin Polypeptide in Rice Seeds", *Plant Mol., 12,* 123–130 (1989).

McCabe, et al., "Stable Transformation of Soybean (*Glycine max*) by Particle Acceration", *Bio/Technol, 6,* 923–926 (1988).

C. McDaniel, et al., "Cell–Lineage Patterns in the Shoot Meristem of the Germinating Maize Embryo", *Planta, 175,* 13–22 (1988).

M. Meadows, "Characterization of Cells and Protoplasts of the B73 Maize Cell Line", *Plant Sci. Lett., 28,* 337–348 (1982/83).

R. Mendel, et al., "Delivery of Foreign Genes to Intact Barley Cell by High–Velocity Microprojectiles", *Theor. Appl. Genet. 78,* 31–34 (1989).

B. V. Milborrow, "Abscisic Acid and Other Hormones", In: *The Physiology and Biochemistry of Drought Resistance in Plants,* Paleg, L.G. et al.(eds). Academic Press, New York, 347–388, (1981).

Morikawa, et al., "Gene Transfer into Intact Plant Cells by Electroporation Through Cell Walls and Membranes", *Gene, 41,* 121 (1986).

S. Morocz, et al., "An Improved System to Obtain Fertile Regenerants via Maize Protoplasts Isolated From a Highly Embryonic Suspension Culture", *Theor. Appl. Genet., 80,* 721–726 (1990).

S. Morocz, et al., "Two Approaches to Rendering *Zea mays* L. Applicable to Tissue Culture Manipulations", *Abstracts VIIth Int. Cong. on Plant Tissue and Cell Culture, Amsterdam A1–102,* Abstract No. 209, p. 190 (1990).

T. Murakami, et al., "The Bialaphos Biosynthetic Genes of *Streptomyces hygroscopicus:* Molecular Cloning and Characterization of the Gene Cluster", *Mol. Gen. Genet. 205,* 42–50, (1986).

T. Murashige, et al., "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures", *Physiol Plant, 15,* 473–497 (1962).

H. L. Murphy, "New Dekalb–Pfizer Seed Chief to Harvest R & D Breakthroughs", *Crain's Business Weekly,* pp. 38–39 (1990).

E. E. Murray, et al., "Codon usage in plant genes", *Nuc. Acids Res., 17,* 477–498 (1989).

T. Nelson, "New Horses for Monocot Gene Jockeys", *The Plant Cell, 2,* 589 (1990).

R. S. Nelson, "Virus Tolerance, Plant Growth, and Field Performance of Transgenic Tomato Plants Expressing Coat Protein from Tobacco mosaic Virus", *Bio/Technol, 6,* 403–409 (1988).

M. Neuffer, et al., "Maize for Biological Research", *Plant Molec. Biol. Assoc.,* 19–30, (1988).
J. Odell, et al., "Identification of DNA Sequences Required for Activity of the Caulliflower Mosaic Virus 35S Promoter", *Nature, 313,* 810–811 (1985).
Y. Ohta, et al., "High–Efficiency Genetic Transformation of Maize by a Mixture of Pollen and Exogenous DNA", *Pro. Nat. Acad. Sci. USA, 83,* 715–719 (1986).
Y. Okta, et al., "Gene Manifestation of Exogenous DNA Applied to Self–Propagating Stigma (Gene Action Revealed in the M1 and M2 Generations from Self–Pollination Applying Exogenous DNA", *Jap. J. Breed., 30,* 184–185 (1980).
P. Ozias–akins, et al., "In vitro regeneration and genetic manipulation of grasses", *Physiol. Plant., 73,* 565–569 (1988).
P. Ozias–akins, et al., "Progress and Limitations in the Culture of Cereal Protoplasts", *Trends in Biotechnol, 2,* 119–123 (1984).
W. B. Parker, et al., "Selection and Characterization of Sethoxydim–Tolerant Maize Tissue Cultures", *Plant Physiol., 92,* 1220–1225 (1990).
K. Pederson, et al., "Sequence Analysis and Characterization of a Maize Gene Encoding a High Sulfure Zein Protein of M1 15,000", *J. Biol. Chem, 261,* 6279–6284 (1986).
R. L. Phillips, et al., "Cell/Tissue Culture and In Vitro Manipulation", In: *Corn and Corn Improvement,* 3rd edition, Sprague, G.F., et al, (eds.). Agronomy Soc. Amer., pp. 345–387 (1988).
R. L. Phillips, et al., "Elevated Protein–Bound Methionine in Seeds of a Maize Line Resistant to Lysine Plus Threonine", *Cereal Chem, 62,* 213–218 (1985).
J. Poehlman, "Breeding Corn (Maize)", In: *Breeding Field Crops, 3rd edition,* AVI Publishing Co., Westport CN, pp. 452 (1986).
J. Poehlman, "Breeding Corn (Maize)", In: *Breeding Field Crops, 3rd edition,* AVI Publishing Co., Westport CN, pp. 469–471, 477–481 (1986).
I. Potrykus, et al., "Callus formation from Cell Culture Protoplasts of Corn (*Zea mays* L.)." *Theor. Appl. Genet.,* 54, 209–214 (1996).
I. Potrykus, et al., "Callus formation from stem protoplasts of corn (*Zea mays* L.)", *Mol. Gen. Genet. 156,* 347–350 (1977).
I. Potrykus, "Gene Transfer to Cereals: An Assessment", *Bio/Technol., 8,* 53–542 (Jun. 1990).
I. Potrykus, "Gene Transfer to Cereals: Assessment", *Trends Biotechnol., 7,* 269–273 (Oct., 1989).
I. Potrykus, "Gene Transfer to Plants: Assesment and Perspectives", *Physiol. Plant, 79,* 125–134 (1990).
Potter, et al., "Enchancer–Dependent Expression of Human k Immunoglobulin Genes Introduced into Mouse Pre–B Lymphocytes by Electroporation", *Pro. Nat. Acad. Sci. USA,* 81, 7161 (1984).
L. M. Prioli, et al., "Plant Regeneration and Recovery of Fertile Plants from Protoplasts of Maize (*Zea mays* L)", *Bio/Technol, 7,* 589–594 (Jun., 1989).
K. J. Puite, et al., "Electrofusion a Simple and Reproducible Technique in Somatic Hybridization of *Nicotiana plumbaginifolia* mutants", *Plant Cell Rep., 4,* 274–276 (1985).
C. A. Rhodes, "Corn: From Protoplasts to Fertile Plants", *Bio/Technol., 7,* 548 (Jun., 1989).
C. A. Rhodes, et al., "Genectically Transformed Maize Plants from protoplasts", *Science, 240,* 204–207 (Apr. 8, 1988).
C. A. Rhodes, et al., "Plant Regeneration from Protoplasts Isolated from Embryogenic Maize Cell Cultures", *Bio/Technol., 6,* 56–60 (Jan. 1988).
F. Richaud, et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli* dapA Gene", *Biol. Abstracts, 82,* p. AB–391, Abstract No. 3396 (1986).
F. Richaud, et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli* dapA Gene", *J. Bacteriol, 166,* 297–300 (1986).
D. S. Robertson, "Loss of Mu Mutator Activity when Active Mu Systems are Transferred to Inbred Lines", *Maize Genetics Coop. Newsletter, 60,* 10 (1986).
M. C. Ross, et al., "Transient and Stable Transgenic Cells and Calli of Tobacco and Maize Following Microprojectile Bombardment", *J. Cell. Biochem., 13D,* p. 268, Abstract No. M149 (1989).
S. V. Sahi, et al., "Metabolites in Maize Which Affect Virulence Induction in *Agrobacterium tumefaciens", Plant Physiol, Supplement,* p. 86, Abstract No. 514 (1989).
J. C. Sanford, et al., "Attempted Pollen–Mediated Plant Transformation Employing Genomic Donor DNA", *Theor. Appl. Genet., 69,* 571–574 (1985).
J. C. Sanford, "Biolistic Plant Transformation", *Physiol. Plant., 79,* 206–209 (1990).
J. C. Sanford, et al., "Delivery of Substances into Cells and Tissues Using a Particle Bombardment Processs", *Particulate Sci. Technol, 5,* 27–37 (1987).
J. C. Sanford, "The Biolistic Process", *Trends Biotechnol, 6,* 299–302 (1988).
Sass, "Morphology: Development of the Caryopsis", In: *Corn and Corn Improvement, 2nd edition,* Sprauge, G.F., (ed), American Soc. Agronomy, p. 89, 98 (1977).
A. Schmidt, et al., "Media and environmental effects of phenolics production from tobacco cell cultures", *Chem. Abstracts., 110,* p. 514, Abstract No. 230156z (1989).
K. Shigekawa, et al., "Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Intoduction of Macromoleucles into Cells", *BioTechniques, 6,* 742–751 (1988).
R. D. Shillito, et al., "High Efficiency Direct Gene Transfer to Plants", *Bio/Technol, 3,* 1099 (1985).
R. D. Shillito, et al., "Regeneration of Fertile Plants From Protoplasts of Elite Inbred Maize", *Bio/Technol, 7,* 581–587 (Jun., 1989).
K. Shimamoto, et al., "Fertile Transgenic Rice Plants Regenerated from Transformed Protoplasts", *Nature, 338,* 274–278 (1989).
M. A. Shotwell, et al., "The Biochemistry of Plants—A Comprehensive Treatise", *The Biochemistry of Plants, vol. 15,* Marcus, A., (ed), Academic Press, Inc, San Diego, pp. 297–345 (1989).
R. Smith, et al., "Shoot apex explant from tranformation", *Plant Physiology (Suppl). 86,* 108, Abstract No. 646 (1988).
X. Soberon, et al., "Construction and Characterization of New Cloning Vehicles, IV. Deletion Derivatives of pBR322 and pBR325", *Gene, 9,* 287–305 (1980).
Spencer, et al., "Bialaphos Selection of Stable Transformations from Maize Cell Culture", *Theor. Appl. Genet., 79,* 625–631 (May, 1990).
T. M. Spencer, et al., "Fertile Transgenic Maize", Abstracts, *7th Annual Meeting, Mid Atlantic Plant Mol. Biol. Soc.,* p. 30 (1990).

T. M. Spencer, et al., "Selection of Stable Transformants from Maize Suspension Cultures using the Herbicide Bialaphos", Poster presentation, *FASEB Plant Gene Expression Conference,* Copper Mountain, Colorado (Aug. 8, 1989).

Sprague, et al., "Corn Breeding", In: *Corn and Corn Improvement,* Sprauge, G. F. (ed.). American Society of Agronomy, Inc, Madison, WI, pp. 305, 320–323 (1977).

M. Sugiyama, et al., "Use of the Tyrosinase Gene from Streptomyces to Probe Promoter Sequences for *Escherichia coli", Plasmid, 23,* 237–251 (1990).

C. Thompson, et al., "Characterization of the Herbicide–Resistance Gene bar from *Streptomyces hygroscopicus", EMBO J., 6,* 2519–2523 (1987).

D. T. Tomes, et al., "Transgenic Tobacco Plants and their Progeny Derived by Microprojectile Bombardment of Tobacco Leaves", *Plant Mol. Biol., 14,* 261–268 (Feb., 1990).

D. Twell, et al., "Transient Expression of chimeric genes delivered into Pollen by Microprojectile Bombardment", *Plant Physiol, 91,* 1271–1274 (1989).

E. Ulian, et al., "Transformation of Plants via the Shoot Apex", *In Vitro Cell Dev. Biol., 9,* 951–954 (1988).

S. Usami, et al., "Absence in Monocotyledonous Plants of the Diffusible Plant Factors including T–DNA Circularization and vir Gene Expression in Agrobacterium", *Mol. Gen. Genet, 209,* 221–226 (1987).

I. K. Vasil, et al., "Culture of Protoplasts Isolated from Embryogenic Cell Suspension Cultures of Sugarcane and Maize", *IAPTC Abstracts,* 443, (1986).

I. K. Vasil, et al., "Isolation and Maintenance of Embryogenic Cell Suspension Cultures of Gramineae", *Cell Culture and Somatic Cell Genetics of Plants, vol. I,* Academic Press, pp. 152–158 (1984).

V. Vasil, et al., "Plant Regeneration from Friable Embryonic Callus and Cell Suspension Cultures of *Zea mays* L", *J. Plant Physiol, 124,* 399–408 (1986).

V. Walbot, et al., "Molecular genetics of corn", In: *Corn and Corn Improvement, 3rd edition,* Sprague, G.F., et al,(eds). American Soc. Agronomy, Madison, WI, pp. 389–430 (1988).

C. Waldron, et al., "Resistance to Hygromycin B", *Plant Mol. Biol., 5,* 103–108 (1985).

Y. Wang, et al., "Transient Expression of Foreign Genes in Rice, Wheat, and Soybean Cells following particle bombardment", *Plant Mol. Biol. 11,* 433–439 (1988).

K. Weising, et al., "Foreign Genes in Plants: Transfer, Structure, Expression and Applicatioins", *Ann. Rev. Genet., 22,* 421–478 (1988).

J. White, et al., "A Cassette Containing the bar Gene of *Streptomyces hygroscopicus:* a Selectable Marker for Plant Transformation", *Nuc. Acid. Res., 18,* 1062 (1989).

H. Yang, et al., "Production of Kanamycin Resistant Rice Tissues Following DNA Uptake into Protoplasts", *Plant Cell Rep., 7,* 421 (1988).

C. Yanisch–perron, et al., "Improved M13 Phage Vectors and Host Strains: Nucleotide Sequences of the M13mp18 and pUC19 Vectors", *Gene, 33,* 103–119 (1985).

Y. Yugari, et al., "Coordinated End–Product Inhibition in Lysine Synthesis in *Escherichia coli.", Biochem. Biophys. Acta. 62,* 612–614 (1962).

Vasil, V., et al., "Regeneration of Plants from Embryogenic Suspension Culture Protoplasts of Wheat (*Triticum aestivum* L.)", *Bio/Technology, 8,* 429–434 (May 1990).

Datta, S.K., et al., "Genetically Engineered Fertile Indica–rice Recovered from Protoplasts", *Bio/Technology, 8,* 736–740 (Aug. 1990).

Abe, K., et al., "Molecular Cloning of a Cysteine Proteinase Inhibitor of Rice (Oryzacystatin)", *The Journal of Biological Chemistry, 262,* 16793–16797 (Dec. 15, 1987).

Anderson, J.M., et al., "The Encoded Primary Sequence of a Rice Seed ADP–glucose Pyrophosphorylase Subunit and Its Homology to the Bacterial Enzyme", *The Journal of Biological Chemistry, 264,* 12238–12242 (Jul. 25, 1989).

Bol, J.F., et al., "Plant Pathogenesis–Related Proteins Induced by Virus Infection", *Annu. Rev. Phytopathol., 28,* 113–138 (1990).

Depicker, A.G., et al., "A Negative Selection Scheme for Tobacco Protoplast–Derived Cells Expressing the T–DNA Gene 2", *Plant Cell Reports, 7,* 63–66 (1988).

Domoney, C., et al., "Cloning and Characterization of Complementary DNA for Convicilin, a Major Seed Storage Protein in *Pisum sativum* L.", *Planta, 159,* 446–453 (1983).

Dunn, G.M., et al., "Inheritance of Cyclic Hydroxamates in *Zea mays* L.", *Can. J. Plant Sci., 61,* 583–593 (Jul. 1981).

Gepts, P., et al., "Enhanced Available Methionine Concentration Associated with Higher Phaseolin Levels in Common Bean Seeds", *Theor. Appl. Genet., 69,* 47–53 (1984).

Guerrero, F.D., et al., "Turgor–Responsive Gene Transcription and RNA Levels Increase Rapidly When Pea Shoots are Wilted. Sequence and Expression of Three Inducible Genes", *Plant Mol. Biol., 15,* 11–26 (1990).

Hu, N.–T., et al., "Primary Structure of a Genomic Zein Sequence of Maize", *The EMBO Journal, 1,* 1337–1342 (1982).

Jaworski, J.G., et al., "A Cerulenin Insensitive Short Chain 3–Ketoacyl–Acyl Carrier Protein Synthase in *Spinacia oleracea* Leaves", *Plant Physiol., 90,* 41–44 (1989).

Joseffson, L.–G., et al., "Structure of a Gene Encoding the 1.7 S Storage Protein, Napin, from *Brassica napus", The Journal of Biological Chemistry, 262,* 12196–12201 (Sep. 5, 1987).

Kim, C.–S., et al., "Improvement of Nutritional Value and Functional Properties of Soybean Glycinin by Protein Engineering", *Protein Engineering, 3,* 725–731 (1990).

Malan, C., et al., "Correlation Between CuZn Superoxide Dismutase and Glutathione Reductase, and Environmental and Xenobiotic Stress Tolerance in Maize Inbreds", *Plant Science, 69,* 157–166 (1990).

Marks, M.D., et al., "Nucleotide Sequence Analysis of Zein mRNAs from Maize Endosperm", *The Journal of Biological Chemistry, 260,* 16451–16459 (Dec. 25, 1985).

Montoliu, L., et al., "A Tandem of α–Tubulin Genes Preferentially Expressed in Radicular Tissues from *Zea mays", Plant Molecular Biology, 14,* 1–15 (1989).

Mundy, J., et al., "Selective Expression of a Probable Amylase/Protease Inhibitor in Barley Aleurone Cells: Comparison to the Barley Amylase/Subtilisin Inhibitor", *Planta, 169,* 51–63 (1986).

O'Reilly, D.R., et al., "A Baculovirus Blocks Insect Molting by Producing Ecdysteroid UDP–Glycosyl Transferase", *Science, 245,* 1110–1112 (Sep. 8, 1989).

Smith, I.K., et al., "Properties and Functions of Glutathione Reductase in Plants", *Physiol. Plant., 77,* 449–456 (1989).

Viotti, A., et al., "Each Zein Gene Class Can Product Polypeptides of Different Sizes", *The EMBO Journal, 4,* 1103–1110 (1985).

Werr, W., et al., "Structure of the Sucrose Synthase Gene on Chromosome 9 of *Zea mays* L.", *The EMBO Journal, 4,* 1373–1380 (1985).

Yenofsky, R.L., et al., "Isolation and Characterization of a Soybean (*Glycine max*) Lipoxygenase–3 Gene", *Mol. Gen. Genet., 211,* 215–222 (1988).

Armstrong, C.L., et al., "Genetic and Cytogenetic Variation in Plants Regenerated from Organogenic and Friable, Embryogenic Tissue Culture in Maize", *Crop Science, 28,* 363–369 (1988).

Dure III, L., et al., "Common Amino Acid Sequence Domains Among the LEA Proteins of Higher Plants", *Plant Molecular Biology, 12,* 475–486 (1989).

Hong, B., et al., "Cloning and Characterization of cDNA Encoding a mRNA Rapidly–Induced by ABA in Barley Aleurone Layers", *Plant Molecular Biology, 11,* 495–506 (1988).

Mundy, J., et al., "Abscisic Acid and Water–Stress Induce the Expression of a Novel Rice Gene", *The EMBO Journal, 7,* 2279–2286 (1988).

Flavell, R., et al., "Prospects for Transforming Monocot Crop Plants", *Nature, 307,* 108–109 (Jan. 12, 1984).

Goodman, R.M. et al., "Gene Transfer in Crop Improvement", *Science, 236,* 48–54 (Apr. 3, 1987).

Paszkowski, J., et al., "Direct Gene Transfer to Plants", *The EMBO Journal, 3,* 2717–2722 (1984).

Potrykus, I., et al., "Direct Gene Transfer to Cells of a Graminaceous Monocot", *Mol. Gen. Genet., 199,* 183–188 (1985).

In Vitro Cellular & Developmental Biology, 21, Program Issue: Thirty–Sixth Annual Meeting of the Tissue Culture Association, New Orleans, LA, 88 p. (Mar. 1985).

In Vitro Cellular & Developmental Biology, 23, Program Issue: Thirty–Eighth Annual Meeting of the Tissue Culture Association, Washington, D.C., 93 p. (Mar. 1987).

In Vitro Cellular & Developmental Biology, 24, Program Issue: Thirty–Ninth Annual Meeting of the Tissue Culture Association, Las Vegas, NV, 92 p. (Mar. 1988).

In Vitro Cellular & Developmental Biology, 25, Program Issue: Fortieth Annual Meeting of the Tissue Culture Association, Orlando, Fl, 73 p. (Mar. 1989).

"European Firm Devises Insect–Resistant Plants", *Agricultural Biotechnology News,* 1, 6 (Mar.–Apr. 1986).

"Molecular Strategies for Crop Improvement", *Journal of Cellular Biochemistry,* Supplement 14e, List of Plenary and Poster Sessions, organized by Arntzen, C., et al., for The Keystone Conference on Molecular Strategies for Crop Plant Improvement, held at the 19th UCLA Symposia, 257 (1990).

Abbe, E.C., et al., "The Growth of the Shoot Apex in Maize: Embryogeny", *American Journal of Botany, 41,* 285–293 (Apr. 1954).

Adang, M.J., et al., "Expression of a *Bacillus thuringiensis* Insecticidal Crystal Protein Gene in Tobacco Plants", *Molecular Strategies for Crop Protection,* Arntzen, C.J., et al. (eds.), Alan R. Liss, Inc., New York, 345–353 (1987).

Anderson, P.C., et al., "Herbicide–Tolerant Mutants of Corn", *Genome, 31,* 994–999 (1989).

Angus, T.A., "Implications of Some Recent Studies of *Bacillus thuringiensis*—A Personal Purview", *Proceedings of the 4th International Colloquium on Insect Pathology,* College Park, MD, 183–189 (Aug. 25–28, 1970).

Armaleo, D., et al., "Biolistic Nuclear Tranformation of *Saccharomyces cerevisiae* and Other Fungi", *Curr. Genet., 17,* 97–103 (1990).

Aronson, A.I., et al., "*Bacillus thuringiensis* and Related Insect Pathogens", *Microbiological Reviews, 50,* 1–24 (Mar. 1986).

Aronson, J.N., et al., "Toxic Trypsin Digest Fragment from the *Bacillus thuringiensis* Parasporal Protein", *Applied and Environmental Microbiology, 53,* 416–421 (Feb. 1987).

Barton, K.A., et al., "*Bacillus thuringiensis* δ–Endotoxin Expressed in Transgenic *Nicotiana tabacum* Provides Resistance to Lepidopteran Insects", *Plant Physiol., 85,* 1103–1109 (1987).

Birk, Y., et al., "Separation of a Tribolium–Protease Inhibitor from Soybeans on a Calcium Phosphate Column", *Biochem. Biophys. Acta, 67,* 326–328 (Feb. 12, 1963).

Bishop, D.H., et al., "Genetically Engineered Viral Insecticides—A Progress Report 1986–1989", *Pestic. Sci., 27,* 173–189 (1989).

Boynton, J.E., et al., "Chloroplast Transformation in Chlamydomonas with High Velocity Microprojectiles", *Science, 240,* 1534–1537 (Jun. 10, 1988).

Bryant, J.A., "At Last: Transgenic Cereal Plants from Genetically Engineered Protoplasts", *Trends in Biotechnology, 6,* 291–292 (Dec. 1988).

Burgerjon, A., et al., "Industrial and International Standardization of Microbial Pesticides—I. *Bacillus thuringiensis", Entomophaga, 22,* 121–129 (1977).

Busvine, J.R., *A Critical Review of the Techniques for Testing Insecticides,* Table of Contents, Commonwealth Agricultural Bureaux, Slough, England, iii–xi (1971).

Bytebier, B., et al., "T–DNA Organization in Tumor Cultures and Transgenic Plants of the Monocotyledon *Asparagus officinalis", Proc. Natl. Acad. Sci. USA, 84,* 5345–5349 (Aug. 1987).

Calabrese, D.M., et al., "A Comparison of Protein Crystal Subunit Sizes in *Bacillus thuringiensis", Canadian Journal of Microbiology, 26,* 1006–1010 (Aug. 1980).

Caplan, A., et al., "Introduction of Genetic Material into Plant Cells", *Science, 222,* 815–821 (Nov. 18, 1983).

Chaleff, R.S., "Induction, Maintenance, and Differentiation of Rice Callus Cultures on Ammonium as Sole Nitrogen Source", *Plant Cell Tissue Organ Culture, 2,* 29–37 (1983).

Christou, P., et al., "Inheritance and Expression of Foreign Genes in Transgenic Soybean Plants", *Proc. Natl. Acad. Sci. USA, 86,* 7500–7504 (Oct. 1989).

Cooksey, K.E., "Purification of a Protein from *Bacillus thuringiensis* Toxic to Larvae of Lepidoptera", *Biochem. J., 106,* 445–454 (1968).

De Block, M., et al., "Expression of Foreign Genes in Regenerated Plants and Their Progeny", *EMBO J., 3,* 1681–1689 (1984).

De Block, M., et al., "The Use of Phosphinothricin Resistance as a Selectable Marker in Tobacco Protoplast Transformation", In: *Progress in Plant Protoplast Research,* Proceedings of the 7th International Protoplast Symposium, Wageningen, The Netherlands, Puite, K.J., et al., (eds.), Kluwer Academic Publishers, Dordrecht, 389–392 (Dec. 6–11, 1987).

Denecke, J., et al., "Quantification of Transient Expression Levels of Genes Transferred to Plant Protoplasts by Electroporation", *Progress in Plant Protoplast Research,* Puite, K.J., et al., (eds.), Proceedings of the 7th International Protoplast Symposium, Wageningen, The Netherlands, 337–338 (Dec. 6–11, 1987).

Duncan, D.R., et al., "The Production of Callus Capable of Plant Regeneration for Immature Embryos of Numerous *Zea mays* Genotypes", *Planta, 165,* 322–332 (1985).

Dunleavy, J.M., "*Curtobacterium plantarum* sp. nov. Is Ubiquitous in Plant Leaves and Is Seed Transmitted in Soybean and Corn", *International Journal of Systematic Bacteriology, 39,* 240–249 (Jul. 1989).

Dybvig, K., et al., "Transposition of Gram–Positive Transposon Tn916 in *Acholeplasma laidlawii* and *Mycoplasma pulmonis", Science, 235,* 1392–1394 (Mar. 13, 1987).

Edallo, S., et al., "Chromosomal Variation and Frequency of Spontaneous Mutation Associated with in vitro Culture and Plant Regeneration in Maize", *Maydica, 26,* 39–56 (1981).

Fast, P.G., et al., "*Bacillus thuringiensis* δ–Endotoxin: Evidence that Toxin Acts at the Surface of Susceptible Cells", *Experientia, 34,* 762–763 (1978).

Faust, R.M., et al., "Bacteria and Their Toxins as Insecticides", In: *Microbial and Viral Pesticides,* Kurstak, E., (ed.), Marcel Dekker, Inc., New York, 75–208 (1982).

Finkle, B.J., et al., "Growth and Regeneration of Alfalfa Callus Lines After Freezing in Liquid Nitrogen", *Plant Science, 42,* 133–140 (1985).

Finney, D.J., In: *Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve,* iii–ix (1952).

Fischoff, D.A., et al., "Insect Tolerant Transgenic Tomato Plants", *Bio/technology, 5,* 807–812 (1987).

Fukoto, T.R., "Physicochemical Aspects of Insecticidal Action", In: *Insecticidal Biochemistry and Physiology,* Wilkinson, C.F., (ed.), Plenum Press, New York, 397–428 (1976).

Gallagher, S., "Progress and Promise of the Particle Gun", *Ag Biotechnology News, 6,* 12–13 (Mar.–Apr. 1989).

Gallie, D.R., et al., "The 5'–leader Sequence of Tobacco Mosaic Virus RNA Enhances the Expression of Foreign Gene Transcripts in Vitro and in Vivo", *Nucleic Acids Research, 15,* 3257–3273 (1987).

Gatehouse, A.M.R., et al., "Assessment of the Antimetabolic Effects of Trypsin Inhibitors from Cowpea (*Vigna unguiculata*) and Other Legumes on Development of the Bruchid Beetle *Callosobruchus maculatus", J. Sci. Food Agric., 34,* 345–350 (1983).

Genovesi, A.D., et al., "Embryogenesis in Callus Derived from Rice Microspores", *Plant Cell Reports, 1,* 257–260 (1982).

Georghiou, G.P., et al., "Factors Influencing the Evolution of Resistance", In: *Pesticide Resistance: Strategies and Tactics for Management,* Committee on Strategies for the Management of Pesticide Resistant Pest Populations, Board on Agriculture, National Research Council, National Academy Press, Washington, D.C., 157–169 (1986).

Gerlach, W.L., "Genetic Engineering: Its Place in Plant Breeding", In: *Plant Breeding and Genetic Engineering,* Zakri, A.H., (ed.), Society for the Advancement of Breeding Researches in Asia and Oceania, Bangi, Malaysia, 269–277 (1988).

Goldfarb, B., et al., "Transient Expression of Microprojectile–Introduced DNA in Douglas–Fir", *J. Cell. Biochem., 13D,* Abstract No. M121, p. 259 (1989).

Goldman, S.L., et al., "Transformation of *Zea mays* by *Agrobacterium tumefaciens:* Evidence for Stable Genetic Alterations", *Journal of Cellular Biochemistry, 11B,* Abstract No. F 202, p. 26 (1987).

Gordon, P.N., et al., "Plant Regeneration from Tissue Cultures of Maize", *Maize Genetics Cooperation Newsletter, 51,* 79–80 (Mar. 1, 1977).

Green, C.E., "New Developments in Plant Tissue Culture and Plant Regeneration", In: *Basic Biology of New Developments in Biotechnology,* Hollaender, A., et al., (eds.), Plenum Press, New York, 195–209 (1983).

Green, C.E., "Somatic Embryogenesis and Plant Regeneration from the Friable Callus of *Zea mays", Proceedings of the 5th International Congress on Plant Tissue & Cell Culture,* Tokyo, Japan, 107–108 (1982).

Haccius, B., "Question of Unicellular Origin of Non–Zygotic Embryos in Callus Cultures", *Phytomorphology, 28,* 74–81 (1978).

Harms, C.T., et al., "Regeneration of Plantlets from Callus Cultures of *Zea mays* L.", *Z. Ptlanzenzuchtg, 77,* 347–351 (1976).

Hartree, E.F., "Determination of Protein: A Modification of the Lowry Method that Gives Linear Photometric Response", *Analytical Biochemistry, 48,* 422–427 (1972).

Harvey, W.R., et al., "Potassium Ion Transport ATPase in Insect Epithelia", *J. Exp. Biol., 106,* 91–117 (1983).

Heimpel, A.M., et al., "Recent Advances in the Knowledge of Some Bacterial Pathogens of Insects", *Proceedings of the Tenth International Congress of Entomology, vol. 4,* 711–722 (1956).

Heimpel, A.M., et al., "The Site of Action of Crystalliferous Bacteria in Lepidoptera Larvae", *Journal of Insect Pathology, 1,* 152–170 (1959).

Hernalsteens, J.–P., et al., "An Agrobacterium–Tranformed Cell Culture from the Monocot *Asparagus Officinalis", The EMBO Journal, 3,* 3039–3041 (Dec. 1984).

Hibberd, K.A., "Induction, Selection, and Characterization of Mutants in Maize Cell Cultures", In: *Cell Culture and Somatic Cell Genetics of Plants, vol. 1,* Vasil, I.K., (ed.), Academic Press, Inc., Orlando, FL, 571–576 (1984).

Hickle, L.A., et al., "Analytical Chemistry of *Bacillus thuringiensis:* An Overview", *In: Analytical Chemistry of Bacillus thuringiensis,* Hickle, L.A., et al., (eds.), Developed from a Symposium Sponsored by the Division of Agrochemicals at the 198th National Meeting of the American Chemical Society, Miami Beach, FL, vii–ix, 1–8 (Sep. 10–15, 1989).

Hilder, V.A., et al., "A Novel Mechanism of Insect Resistance Engineered into Tobacco", *Nature, 330,* 160–163 (Nov. 12, 1987).

Hodges, T.K., et al., "Genotype Specificity of Somatic Embryogenesis and Regeneration in Maize", *Bio/technology, 4,* 219–223 (Mar. 1986).

Hodges, T.K., et al., "Regeneration of Maize", In: *Biotechnology in Plant Science,* Zaitlin, M., et al., (ed.), Academic Press, Inc., Orlando, FL, 15–33 (1985).

Hoekema, A., et al., "Codon Replacement in the PGK1 Gene of *Saccharomyces cerevisiae:* Experimental Approach to Study the Role of Biased Codon Usage in Gene Expression", *Molecular and Cellular Biology, 7,* 2914–2924 (Aug. 1987).

Hofmann, C., et al., "Binding of the Delta Endotoxin from *Bacillus thuringiensis* to Brush–Border Membrane Vesicles of the Cabbage Butterfly (*Pieris Brassicae*)", *Eur. J. Biochem., 173,* 85–91 (1988).

Hofmann, C., et al., "Specificity of *Bacillus thuringiensis* δ–Endotoxins is Correlated with the Presence of High–Affinity Binding Sites in the Brush Border Membrane of Target Insect Midguts", *Proc. Natl. Acad. Sci. USA,* 85, 7844–7848 (Nov. 1988).

Höfte, H., et al., "Monoclonal Antibody Analysis and Insecticidal Spectrum of Three Types of Lepidopteran–Specific Insecticidal Crystal Proteins of *Bacillus thuringiensis", Applied and Environmental Microbiology, 54,* 2010–2017 (Aug. 1988).

Höfte, H., et al., "Structural and Functional Analysis of a Cloned Delta Endotoxin of *Bacillus thuringiensis berliner* 1715", *Eur. J. Biochem., 161,* 273–280 (1986).

Hollingworth, R.M., "The Biochemical and Physiological Basis of Selective Toxicity", In: *Insecticidal Biochemistry and Physiology,* Wilkinson, C.F., (ed.), Plenum Press, New York, 431–506 (1976).

Horsch, R.B. et al., "A Simple and General Method for Transferring Genes into Plants", *Science, 227,* 1229–1231 (Mar. 8, 1985).

Huber, H.E., et al., "*Bacillus thuringiensis* δ–Endotoxin: Composition and Activation", In: *Pathogenesis of Invertebrate Microbial Diseases,* Davidson, E.W., (ed.), Allanheld, Osmun & Co. Publishers, Inc., Totowa, NJ, 209–234 (1981).

Huber–Lukac, M., et al., "Characterization of Monoclonal Antibodies to a Crystal Protein of *Bacillus thuringiensis* subsp. *kurstaki", Infection and Immunity, 54,* 228–232 (Oct. 1986).

Imbrie–Milligan, C.W., et al., "Microcallus Formation from Maize Protoplasts Prepared from Embryogenic Callus", *Planta, 168,* 395–401 (1986).

Jarrett, P., "Potency Factors in the delta–Endotoxin of *Bacillus thuringiensis* var. *aizawi* and the Significance of Plasmids in their Control", *Journal of Applied Bacteriology, 58,* 437–448 (1985).

Johnson, D.E., "Toxicity of *Bacillus thuringiensis* Entomocidal Protein Toward Cultured Insect Tissue", *Journal of Invertebrate Pathology, 38,* 94–101 (1981).

King, P., et al., "Maize", In: *Handbook of Plant Cell Culture, vol. 2,* Sharp, W.R., et al., (eds.), Macmillan Publishing Company, New York, 69–91 (1984).

Klein, T.M., et al., "Advances in Direct Gene Transfer into Cereals", *In: Genetic Engineering: Principles and Methods, vol. 11,* Setlow, J.K., (ed.), Plenum Publishing Corp., New York, 13–31 (1989).

Klein, T.M., et al., "Particle Gun Technology: A Novel Method for the Introduction of DNA into Living Cells", *Program and Abstracts for an International Symposium: "Biotechnology in Plant Science: Relevance to Agriculture in the Eighties",* Poster, #28, Ithaca, NY, 25 (Jun. 23–27, 1985).

Klein, T.M., et al., "Stable Genetic Transformation of Intact Nicotiana Cells by the Particle Bombardment Process", *Proc. Natl. Acad. Sci. USA, 95,* 5502–5505 (Nov. 1988).

Knowles, B.H., et al., "Characterization and Partial Purification of a Plasma Membrane Receptor for *Bacillus thuringiensis* var. *kurstaki* Lepidopteran–Specific δ–Endotoxin", *J. Cell Sci., 83,* 89–101 (1986).

Knowles, B.H., et al., "Lectin–Like Binding of *Bacillus thuringiensis* var. *kurstaki* Lepidopteran–Specific Toxin is an Initial Step in Insecticidal Action", *FEBS Letters, 168,* 197–202 (Mar. 1984).

Langridge, W.H., et al., "Electric Field Mediated DNA Transformation in Plant Protoplasts", *Program and Abstracts for an International Symposium: "Biotechnology in Plant Science: Relevance to Agriculture in the Eighties",* Ithaca, NY, Poster #30, p. 25 (Jun. 23–27, 1985).

Leason, M., et al., "Inhibition of Pea Leaf Glutamine Synthetase by Methionine Sulphoximine, Phosphinothricin and Other Glutamate Analogues", *Biochemistry, 21,* 855–857 (1982).

Lee, B., "Cereal Transformation", *Planta Today,* 9–11 (Jan.–Feb. 1989).

Lörz, H., et al., "Gene Transfer to Cereal Cells Mediated by Protoplast Transformation", *Mol. Gen. Genet., 199,* 178–182 (1985).

Lowe, K., et al., "Plant Regeneration via Organogenesis and Embryogenesis in the Maize Inbred Line B73", *Plant Science, 41,* 125–132 (1985).

Luckow, V.A., et al., "Trends in the Development of Baculovirus Expression Vectors", *Bio/Technology, 6,* 47–55 (Jan. 1988).

Lüthy, P., "Insecticidal Toxins of *Bacillus thuringiensis", FEMS Microbiology Letters, 8,* 1–7 (1980).

Mangano, M.L., et al., "Long–Term Cold Storage of Regenerable Maize Callus", *In Vitro Cellular and Developmental Biology, 25,* Abstract No. 224, p. 66A (Mar. 1989).

Merryweather, A.T., et al., "Construction of Genetically Engineered Baculovirus Insecticides Containing the *Bacillus thuringiensis* subsp. *kurstaki* HD–73 Delta Endotoxin", *Journal of General Virology, 71,* 1534–1544 (1990).

Molnar, S.J., et al., "Initiation of Totipotent Tissue Cultures from Undeveloped Axillary and Secondary Ears", *Maize Genetics Cooperation Newsletter, 54,* 52–53 (Mar. 31, 1980).

Murphy, D.W., et al., "*Bacillus thuringiensis* Enzyme–Digested Delta Endotoxin: Effect of Cultured Insect Cells", *Science, 194,* 954–956 (Nov. 26, 1976).

Nishiitsutsuji–Uwo, J., et al., "Mode of Action of *Bacillus thuringiensis* δ–Endotoxin: Effect on TN–368 Cells", *Journal of Invertebrate Pathology, 34,* 267–275 (1979).

Ochatt, S.J., et al., "Selection of Salt/Drought Tolerance using Isolated Protoplasts and Protoplast–Derived Calli of Colt Cherry (*Prunus avium* x *pseudocerasus*)", In: *Progress in Plant Protoplast Research,* Puite, K.J., et al., (eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, p. 391–392 (1988).

Oeda, K., et al., "Formation of Crystals of the Insecticidal Proteins of *Bacillus thuringiensis* subsp. *aizawai* IPL7 in *Escherichia coli", Journal of Bacteriology, 171,* 3568–3571 (Jun. 1989).

Park, W.D., et al., "High–Level, Sucrose–Inducible Expression of a Chimeric Patatin–GUS Gene In Leaf Explants of Transgenic Tobacco Plants", *Journal of Cellular Biochemistry, 13D,* Abstract No. M 343, p. 310 (Mar. 27–Apr. 7, 1989).

Perlak, F.J., et al., "Expression of *Bacillus thuringiensis* Proteins in Transgenic Plants", *In: Biotechnology, Biological Pesticides and Novel Plant–Pest Resistance for Insect Pest Management,* Roberts, D.W., et al., (eds.), Insect Pathology Resource Center, Boyce Thompson Institute for Plant Research, Cornel University, Ithaca, NY, 77–81 (1988).

Poehlman, J.M., et al., In: *Breeding Field Crops, 3rd Edition,* AVI Publishing Company, Inc., Westport, CT, 149–152 (1987).

Poethig, R.S., "Maize—The Plant and Its Parts", In: *Maize for Biological Research,* Sheridan, W.F., (ed.), Plant Molecular Biology Association., Charlottesville, VA, 9–18 (1982).

Potrykus, I., et al., "Direct Gene Transfer: State of the Art and Future Potential", *Plant Molecular Biology Reporter, 3,* 117–128 (Summer 1985).

Randolph, L.F., et al., "Developmental Morphology of the Caryopsis in Maize", *Journal of Agricultural Research, 53,* 881–916 (Dec. 15, 1936).

Rhodes, C.A., et al., "Cytogenetic Stability of Aneuploid Maize Tissue Cultures", *Can. J. Genet. Cytol., 28,* 374–384 (1986).

Rhodes, C.A., et al., "Factors Affecting Tissue Culture Initiation from Maize Tassels", *Plant Science, 46,* 225–232 (1986).

Rice, T.B., "Tissue Culture Induced Genetic Variation in Regenerated Maize Inbreds", *Proceedings of the 37th Annual Corn & Sorghum Industry Research Conference,* 148–162 (1982).

Rosahl, S., et al., "Expression of a Tuber–Specific Storage Protein In Transgenic Tobacco Plants: Demonstration Of An Esterase Activity", *EMBO J, 6,* Press Limited, Oxford, England, 1155 (1987).

Roth, B.A., et al., "Genetic Regulation of Transient Expression of Maize Anthocyanin Pathway Genes Introduced into Intact Maize Tissues by Microprojectile Bombardment", *Journal of Cellular Biochemistry, 13D,* Abstract No. M 344, p. 310 (Mar. 27–Apr. 7, 1989).

Roush, R.T., et al., "Ecological Genetics of Insecticidal and Acaricide Resistance", *Ann. Rev. Entomol., 32,* 361–380 (1987).

Ryan, A.J., et al., "The Expression of the Napin Gene Under the Control of Its Own Promoter in Transgenic Tobacco Plants", *Journal of Cellular Biochemistry, 13D,* Abstract No. M 345, p. 310 (Mar. 27–Apr. 7, 1989).

Sanford, J.C., "The Biolistic Process", *Plant Physiology, 89,* Abstract No. 9, p. 2 (Apr. 1989).

Sanford, J.C., et al., "Delivery of DNA into Regenerable Tissues of Monocots, Using High–Velocity Microprojectiles", Grant Application No. .86–0183, United States Department of Agriculture, Science and Education, 57 p. (Feb. 27, 1986).

Sass, J.E., "Comparative Leaf Number in the Embryos of Some Types of Maize", *Iowa State Coll. J. Sci., 25,* 509–512 (1951).

Schafer, W., et al., "T–DNA Integration and Expression in a Monocot Crop Plant after Induction of Agrobacterium", *Nature, 327,* 529–532 (Jun. 11, 1987).

Schardl, C.L., et al., "Design and Construction of a Versatile System for the Expression of Foreign Genes in Plants", *Gene, 61,* 1–11 (1987).

Schnepf, H.E., et al., "Delineation of a Toxin–Encoding Segment of a *Bacillus thuringiensis* Crystal Protein Gene", *The Journal of Biological Chemistry, 260,* 6273–6280 (1985).

Shaner, D.L., et al., "Mechanism of Action of the Imidazolinones and Cell Culture Selection of Tolerant Maize", In: *Biotechnology in Plant Sciences,* Zaitlin, M., et al., (eds.), Academic Press, Orlando, FL, 287–299 (1985).

Sharman, B.C., "Developmental Anatomy of the Shoot of *Zea mays* L.", *Annals of Botany, VI,* 246–281 (Apr. 1942).

Shields, R., "Towards Insect–Resistant Plants", *Nature, 328,* 12–13 (Jul. 2, 1987).

Shivakumar, A.G., et al., "Vegetative Expression of the δ–Endotoxin Genes of *Bacillus thuringiensis* subsp. *kurstaki* in *Bacillus subtilis", Journal of Bacteriology, 166,* 194–204 (Apr. 1986).

Smith, G.E., et al., "Molecular Engineering of the *Autographa californica* Nuclear Polyhedrosis Virus Genome: Deletion Mutations Within the Polyhedrin Gene", *Journal of Virology, 46,* 584–593 (May 1983).

St. Julian, G., et al., "Bacteria, Spirochetes, and Rickettsia as Insecticides", *Annals of the New York Academy of Sciences, 217,* 65–75 (1973).

Stalker, D.M., et al., "Herbicide Resistance in Transgenic Plants Expressing a Bacterial Detoxification Gene", *Science, 242,* 419–422 (Oct. 21, 1988).

Stolle, C.A., et al., "Cellular Factor Affecting the Stability of β–globulin mRNA", *Gene, 62,* 65–74 (1988).

Strauch, E., et al., "Cloning of a Phosphinothricin N–Acetyltransferase Gene from *Streptomyces viridochromogenes* Tu494 and its Expression in *Streptomyces lividans* and *Escherichia coli", Gene, 63,* 65–74 (1988).

Stroo, H.F., et al., "Heterotrophic Nitrification in an Acid Forest Soil and by an Acid–Tolerant Fungus", *Applied and Environmental Microbiology, 52,* 1107–1111 (Nov. 1986).

Suprasanna, P., et al., "Plantlet Regeneration from Glume Calli of Maize (*Zea mays* L.)", *Theor. Appl. Genet., 72,* 120–122 (1986).

Thomas, W.E., et al., "Mechanisms of Action of *Bacillus thuringiensis* var *israelensis* Insecticidal δ–Endotoxin", *FEBS Letters, 154,* 362–368 (Apr. 1983).

Tojo, A., et al., "Dissolution and Degradation of *Bacillus thuringiensis* δ–Endotoxin by Gut Juice Protease of the Silkworm *Bombyx mori", Applied and Environmental Microbiology, 45,* 576–580 (Feb. 1983).

Tomes, D.T., "Cell Culture, Somatic Enbryogenesis and Plant Regeneration in Maize, Rice, Sorghum and Millets", In: *Cereal Tissue and Cell Culture,* Bright, S.W.J., et al., (eds.), Martinus Nijnoff/Dr. W. Junk, Amsterdam, The Netherlands, 175–203 (1985).

Tomes, D.T., "Initiation of Embryogenic Callus Cultures from Immature Embryos of Elite Corn (*Zea mays* L.) Germplasm", *In Vitro, 20,* Abstract No. 146, P. 276 (Mar. 1984).

Tomes, D.T., et al., "The Effect of Parental Genotype on Initiation of Embryogenic Callus from Elite Maize (*Zea mays* L).) Germplasm", *Theor. Appl. Genet., 70,* 505–509 (1985).

Torne, J.M., et al., "Regeneration of Plants from Mesocotyl Tissue Cultures of Immature Embryos of *Zea mays* L.", *Plant Science Letters, 17,* 339–344 (1980).

Vaeck, M., et al., "*Bacillus thuringiensis* Endotoxin Gene Expression and Insect Resistance in Higher Plants", *Pesticide Science, 20,* 319–320 (1987).

Vaeck, M., et al., "Engineering Improved Crops for Agriculture: Protection from Insects and Resistance to Herbicides", In: *Plant Gene Systems and Their Biology,* Key, J.L., et al., (eds.), Alan R. Liss, Inc., New York, 171–181 (1987).

Vaeck, M., et al., "Engineering of Insect Resistant Plants Using a *B. thuringiensis* Gene", *In: Molecular Strategies for Crop Protection,* New York, Alan R. Liss, Inc., 355–366 (1987).

Vaeck, M., et al., "Insect Resistance in Transgenic Plants Expressing *Bacillus thuringiensis* Toxin Gens", *An. Soc. Entomol. Brasil, 16,* 427–435, (1987).

Vaeck, M., et al., "Protein Engineering in Plants: Expression of *Bacillus thuringiensis* Insecticidal Protein Genes", *Cell Culture and Somatic Cell Genetics of Plants, 6,* 425–439, (1989).
Vaeck, M., et al., "Transgenic Plants Protected from Insect Attack", *Nature,* 328, 33–37, (Jul. 2, 1987).
van den Elzen, P.J., et al., "A Chimaeric Hygromycin Resistance Gene as a Selectable Marker in Plant Cells", *Plant Molecular Biology, 5,* 299–302, (1985).
van den Elzen, P.J., et al., "Simple Binary Vectors for DNA Transfer to Plant Cells", *Plant Molecular Biology, 5,* 149–154, (1985).
Van Lammeren, A.A., "Developmental Morphology and Cytology of the Young Maize Embryo (*Zea mays*L.)", *Acta Bot. Neerl., 35,* 169–188 (Aug. 1986).
Vasil, I.K., "Isolation and Culture of Protoplasts of Grasses", *International Review of Cytology, Supplement 16,* Bourne, G.H., et al., (eds.), Academic Press, New York, 79–88 (1983).
Vasil, V., et al., "Histology of Somatic Embryogenesis in Cultured Immature Embryos of Maize (*Zea mays* L.)", *Protoplasma, 127,* 1–8 (1985).
Watson, S.A., "Corn Marketing, Processing and Utilization", *In: Corn and Corn Improvement, 3rd Edition,* Sprague, G.F., et al., (eds.), American Society of Agronomy, Inc., et al., Madison, WI, 881–939 (1988).
Weigel, Jr., R.D., et al.,"Somatic Embryogenesis in Barley", *In Vitro, 20,* Abstract No. 147, p. 277 (Mar. 1984).
Weissinger, A., et al., "Maize Transformation via Microprojectile Bombardment", *In: Genetic Improvements of Agriculturally Important Crops,* Fraley, R.T., et al., (eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 21–25 (1988).
Weissinger, A., et al., "Microprojectile Bombardment for Maize Transformation", *In Vitro Cellular and Developmental Biology, 23,* Program Issue, 38th Annual Meeting of the Tissue Culture Association, Washington, D.C., Abstract No. 254 (Mar. 1987).
Wernicke, W., et al., "Adventitious Embryoid and Root Formation from Rice Leaves", *Z. Pflanzenphysiol. Bd., 103,* 361–365 (1981).
Withers, L., et al., "Proline: A Novel Cryoprotectant for the Freeze Preservation of Cultured Cells of *Zea mays* L.", *Plant Physiology, 64,* 675–678 (1979).
Witt, D.P., et al., "Cytotoxicity of *Bacillus thuringiensis* δ–Endotoxins to Cultured Cf–1 Cells Does Not Correlate with In Vivo Activity Toward Spruce Budworm Larvae", *In: Fundamental and Applied Aspects of Invertebrate Pathology,* Samson, R.A., et al., (eds.), Fourth International Colloquium of Invertebrate Pathology, Wangingen, The Netherlands, 3–6 (Aug. 18–22, 1986).
Wohlleben, W., et al., "Nucleotide Sequence of the Phosphinothricin N–Acetyltransferase Gene from *Streptomyces viridochromogenes* Tü494 and Its Expression in *Nicotania tabacum", Gene, 70,* 25–37 (1988).
Wood, M., "Blast Those Genes!", *Agricultural Research,* 2 p. (Jun. 1989).
"Bullets Transform Plant Cells", *Agricell Report,* 9, 5, (Jul. 1987).
"Shotgunning DNA into Cells", *Genetic Engineering News,* (Jul./Aug. 1987).
H. Ahokas, "Transfection of Germinating Barley Seed Electrophorectically with Exogenous DNA", *Theor. Appl. Genet., 77,* 469–472(1989).
H. Ahokes, "Electrophorectic transfection of cereal grain with exogenous nucleic acid", *Soc., biochem Biophys. Microbio. Fen., Biotieteen Paivat* (*Bioscience Days*), Abstracts, Technical University of Helsinki, Espoo, p. 2 (1989).
S. B. Altenbach, et al., "Cloning and Sequence Analysis of a cDNA Encoding a Brazil Nut Protein Exceptionally Rich in Methionine", *Plant Mol. Biol., 8,* 239–250 (1987).
C. Ampe, et al., "The Amino–Acid Sequence of the 2S Sulphur–Rich from Seed of Brazil Nut (*Bertholletia excelsa* H.B.K.)", *Eur. J. Biochem., 159,* 597–604 (1986).
C. Armstrong, et al., "Genetic and Cytogenetic Variation in Plants Regenerated from Organogenic and Friable Embryogenic Tissue Culture in Maize", *Biol. Abstracts, 85,* Abstract No. 117662 (1988).
Barker, R. F., et al., "Nucleotide Sequence of the T–DNA Region from the *Agrobacterium tumefaciens* Octopone Ti Plasmid pTi15955," *Plant Mol. Biol., 2,* 335–350 (1983).
M. S. Benner, et al., "Genetic Analysis of Methionine–Rich Storage Protein Accumulation in Maize", *Theor. Appl. Genet., 78,* 761–767 (1989).
M. Bevan, et al., "A Chimaeric Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation", *Nature, 304,* 184–187 (1983).
M. Bevan, et al., "Structure and Transcription of the Nopaline Synthase Gene Region of T–DNA", *Nuc. Acids Res., 11,* 369–385 (1983).
G. Booy, et al., "Attempted Pollen–Mediated Transformation of Maize", *J. Plant Physiol., 135,* 319–324 (1989).
J. S. Boyer, et al., "Water Deficits and Photosynthesis," In: *Water Deficits and Plant Growth, vol. IV,* T. T. Kozlowski, (ed.), Academic Press, New York, pp. 153–190 (1976).
Callis, J., et al., "Introns Increase Gene Expression in Cultures Maize Cells," *Genes and Development, 1,* 1183–1200 (1987).
J. Cao, et al., "Transformation of Rice and Maize Using the Biolistic Process", In: *Plant Gene Transfer,* Alan R. Liss, Inc., pp. 21–23 (1990).
V. Chandler, et al., "Two Regulatory Genes of the Maize Anthocyanin Pathway Are Homolgous: Isolation of B Utilizing R Genomic Sequences", *The Plant Cell, 1,* 1175–1183 (1989).
P. Christou, et al., "Cotransformation Frequencies of Foreign Genes in Soybean Cell Cultures", *Theor. Appl. Genet., 79,* 337–341 (1990).
P. Christou, et al., "Stable Transformation of Soybean Callus DNA–Coated Gold Particles", *Plant Physiol., 87,* 671–674 (1988).
Cocking, F., et al., "Gene Transfer in Cereals," *Science,* 236, 1259–1262 (1987).
G. Creissen, et al., "Agrobacterium—and microprojectile—Mediated Viral DNA Delivery into Barley Microspore–Derived Cutures", *Plant Cell Reports, 8,* 680–863 (Apr., 1990).
A. Crossway, et al., "Integrated of foreign DNA following microinjection of tobacco mesophyll protoplasts", *Mol. Gen. Genet., 202,* 179–185 (1986).
M. De Block et al., "Engineering herbicide resistance on plants by expression of a detoxifying enzyme", *EMBO J., 6,* 2513–2518 (1987).
W. De Greef, et al., "Evaluation of herbicide resistance in transgenic crops under field conditions", *Bio/Technol., 7,* 61–64 (1989).

R. Dekeyser, et al., "Evaluation of Selectable Markers for Rice Transformation", *Plant Physiol., 90,* 217–223 (1989).

DeWald, et al., "Plant regeneration from inbred maize suspensions", *VIIth International Congress on Plant Tissue and Cell Culture,* Abstract No. A1–36, p. 12 (Jun. 24–29, 1990).

DeWet, J. R. et al., "Cloning of Firefly Luciferase cDNA and the Expression of Active Luciferase in *Escherichia coli," Proc. Nat. Acad. Sci. USA, 82,* 7870–7873 (1985).

D. A. Evans, et al., "Somaclonal Variation–Genetic Basis and Breeding Applications", *Trends Genet., 5,* 46–50 (1989).

P. Fransz, et al., "Cytodifferentiation during callus initiation and somatic embryogenesis in *Zea mays* L", Ph. D. thesis, U of Wageningen Press, the Netherlands (1988).

J. C. Freeling, et al., "Development Potentials of Maize Tissue Cultures", *Maydica, XXL,* 97–112 (Jul 1977).

Fromm, M. E., et al., "Stable Transformation of Maize after Gene Transfer by Electroporation," *Nature, 319,* 791–793 (1986).

M. E. Fromm, et al., "Expression of Gene Transfected into Monocot and Dicot Plant Cells by Electroporation", *Pro. Nat. Acad. Sci. USA, 82,* 5824–5828 (1985).

W. J. Gordon–kamm, et al., "Stable Tranformation of Embryonic Maize Cultures by Microprojectile Bombardment", *J. Cellular Biochem., 13D,* Abstract No M122, p. 259 (1989).

J. Gould, et al., "Shoot Tip Culture as a Potential Transformation System", *Abstracts, Beltwide Cotton production research conferences,* New Orleans, LA, p. 91 (1988).

Graves, A., et al., "The transformation of *Zea mays* seedlings with *Agrobacterium tumefacians," Plant Mol. Biol., 7,* 43–50 (1986).

Green, C., et al., "Plant Regeneration from Tissue Cultures of Maize," *Crop. Sci., 15,* 417–421 (1975).

Green, C., et al., "Plant Regeneration in Tissue Cultures of Maize," In: *Maize for Biological Research,* Sheridan, W. F., (ed.) Plant Mol. Biol. Assoc., pp. 367–372 (1982).

C. Green, et al., "Somatic Cell Genetic System in Corn", In: *Advances in Gene Technology: Molecular Genetics of Plant and Animals,* Academic Press, Inc., pp. 147–157 (1983).

N. Grimsley, et al., "DNA Transfer from Agrobacterium to *Zea mays* or Brassica by Agroinfection is Dependent on Bacterial Virulence Functions", *Mol. Gen. Genet., 217,* 309–316 (1989).

Gritz, L., et al., "Plasmid–Encoded Hygromycin B Resistance: The Sequence of Hygromycin B Phosphotransferase Gene and Its Expression in *Escherichia coli* and *Saccharomyces cerevisiae," Gene, 25,* 179–188 (1983).

Guilley, H., et al., "Transcription of Cauliflower Mosaic Virus DNA: Detection of_Promoter Sequences, and Characterization of Transcripts," *Cell; 30,* 763–773 (Oct. 1982).

R. M. Hauptman, et al., "Evaluation of Selectable Markers for Obtaining Stable Transformants on the Gramineae", *Plant Physiol., 86,* 602–606 (1988).

L. M. Hoffman, et al., "A Modified Storage Protein is Synthesized, Processed, and Degraded in the Seeds of Transgenic Plants", *Plant. Mol. Biol., 11,* 717–729 (1988).

L. M. Hoffman, et al., "Synthesis and Protein Body Deposition of Maize 15kD Zein in Transgenic Tobacco Seeds", *EMBO J., 6,* 3213–3221 (1987).

P. J. J. Hooykaas, "Transformation of plant cell via Agrobacterium", *Plant Mol. Biol., 13,* 327–336 (1989).

Horn, M., et al., "Transgenic Plants of Orchard Grass (*Dactylis glomerata* L.) from Protoplasts," *Chem. Abstracts, 110,* p. 208, Abstract No. 89869a (1989).

R. Jefferson, "Assaying chimeric genes in plants: the GUS gene fusion system", *Plant Mol. Biol. Rep., 5,* 387–405(1987).

R. Jefferson, et al., "B–Glucuronidase from *Escherichia coli* as a Gene–Fusion Marker", *Proc. Natl. Acad. Sci. USA, 83,* 8447–8451 (1986).

Jefferson, R., "Assaying chimeric genes in plants: the GUS gene fusion system," *Plant Mol. Biol. Rep., 5,* 387–405 (1987).

K. Kamo, et al., "Establishment and Characterization of Long–Term Embryonic Maize Callus and Cell Suspension Cultures", *Plant Sci., 45,* 111–117 (1986).

K. Kamo, et al., "Regeneration of *Zea mays* L. from Embryogenic Callus", *Bot. Gaz., 146,* 327–334 (1985).

K. Kartha, et al., "Transient Expression of Chloramphenicol Acetyl Transferase (CAT) Gene in Barley Cell Cultures and Immature Embryos Through Microprojectile Bombardment", *Plant. Cell. Rep., 8,* 429–432 (1989).

J. Kirhara, et al., "Differential Expression of a Gene for a Methionine–Rich Storage Protein in Maize", *Mol. Gen. Genet., 211,* 477–484 (1988).

J. Kirihara, et al., "Isolation and Sequence of a Gene Encoding a Methionine–rich 10–kD Zein Protein from Maize", *Gene, 71,* 359–370 (1988).

Klein, T., et al., "Transfer of Foreign Genes into Intact Maize Cells with High–Velocity Microprojectiles," *Proc. Nat. Acad. Sci. USA, 85,* 4305–4309 (1988).

Klein, T. M., et al., "Factors Influencing Gene Delivery into *Zea mays* Cells by High Velocity Microprojectiles," *Bio/Technol., 6,* 559–563 (1988).

Klein, T., et al., "Genetic transformation of Maize Cells by Particle Bombardment," *Plant Physiol., 91,* 440–444 (1989).

T. M. Klein, et al., "Genetic Transformation of Maize Cell by Particle Bombardment and the Influence of Mehtylation on Foreign Gene Expression", In: *Gene Manipulation in Plant Improvement II,* Gustafson, J.P., (ed), Plenum Press, NY, pp. 265–266 (1990).

T. M. Klein, et al., "High–Velocity Microprojectiles for Delivering Nucleic Acids to Living Cells", *Nature, 327,* 70–73 (1987).

T. Klein, et al., "Regulation of Anthocyanin Biosynthetic Genes Introduced into Intact Maize Tissue by Microprojectiles", *Pro. Nat. Acad. Sci., USA, 86,* 6682–6685 (1989).

M. Kozak, "Point Mutations Define a Sequence Flanking the AUG Inhibitor Codon that Modulates Translation by Eukaryotic Ribosomes", *Cell, 44,* 283–292 (1986).

P. Lazzeri, et al., "In Vitro Genetic Manipulation of Cereals and Grasses", *Ad. Cell Culture, 6,* 291–293 (1988).

K. Lindsey, et al., "Electroporation of Cells", *Physiologia Plantarum, 79,* 168–172 (1990).

H. Lorz, et al., "Advances in Tissue Cultures and Progress Towards Genetic Transformation of Cereals", *Plant Breeding, 100,* 1–25 (1988).

C. Lu, et al., "Improved Efficiency of Somatic Embryogenesis . and Plant Regeneration on Tissue Cultures of Maize(*Zea mays* L__", *Theor. Appl. Genet., 66,* 285–289 (1983).

S. Ludwig, et al., "A Regulatory Gene as a Novel Visible Marker for Maize Transformation", *Science, 257,* 449–450 (1990).

S. Ludwig et al., "High Frequency Callus Formation from Maize Protoplasts", *Theor. Appl. Genet., 71,* 344–350 (1985).

S. Ludwig, et al., "Lc, a Member of the Maize R Gene Family Responsible for Tissue–Specific Anthocyanin Production, Encodes a Protein Similar to Transcriptional Activators and Contains the myc–Homology Region", *Proc., Nat. Acad. Sci. USA, 86,* 7092–7096 (1989).

S. Ludwig, et al., "Maize R Gene Family: Tissue Specific Helix Loop Proteins", *Cell, 62,* 849–851 (1990).

H. Lutcke, et al., "Selection of AUG Initiation Codons Differs in Plants and Animals", *EMBO J., 6,* 43–48 (1987).

T. Masamura, et al., "cDNA Cloning of an mRNA Encoding a Sulfur–Rich 10 kDa Prolamin Polypeptide in Rice Seeds", *Plant Mol., 12,* 123–130 (1989).

C. McDaniel, et al., "Cell–Lineage Pattens in the Shoot Apical Meristem of the Germinating Maize Embryo", *Planta, 175,* 13–22 (1988).

M. Meadows, "Characterization of Cells and Protoplasts of the B73 Maize Cell Line", *Plant Sci. Lett., 28,* 337–348 (1982/83).

R. Mendel, et al., "Delivery of Foreign Genes to Intact Barley Cell by High–Velocity Microprojectiles", *Theor. Appl. Genet., 78,* 31–34 (1989).

B. V. Milborrow, "Abscisic Acid and Other Hormones," In: *The Physiology and Biochemistry of Drought Resistance in Plants,* Paleg, L. G., et al., (eds.), Academic Press, New York, pp 347–388 (1981).

T. Murakami, et al., "The Bialaphos Biosynthetic Genes of *Streptomyces hygroscopicus:* Molecular Cloning and Characterization of the Gene Cluster", *Mol. Gen. Genet., 205,* 42–50 (1986).

T. Nelson, "New Horses for Monocot Gene Jockeys", *The Plant Cell, 2,* 589 (1990).

M. Neuffer, et al., "Maize for Biological Research", *Plant Molec. Biol. Assoc.,* 19–30 (1988).

J. Odell, et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter", *Nature, 313,* 810–811 (1985).

Y. Ohta, et al., "High–Efficiency Genetic Transformation of Maize by a Mixture of Pollen and Exogenous DNA", *Pro. Nat. Acad. Sci. USA, 83,* 715–719 (1986).

Y. Okta, et al., "Gene Manfestation of Exogenous DNA Applied to Self–Propagating Stigma (Gene Action Revealed in the M1 and M2 Generations from Self–Pollination Applying Exogenous DNA", *Jap. J. Breed., 30,* 184–185 (1980).

P. Ozias–akins, et al., "In vitro regeneration and genetic manipulation of grasses", *Physiol. Plant., 73,* 565–569 (1988).

P. Ozias–akins, et al., "Progress and Limitations in the Culture of Cereal Protoplasts", *Trends in Biotechnol., 2,* 119–123 (1984).

K. Pederson, et al., "Sequence Analysis and Characterization of a Maize Gene Encoding a High Sulfure Zein Protein of M1 15,000", *J. Biol. Chem., 261,* 6279–6284 (1986).

R. L. Phillips, et al., "Cell/Tissue Culture and In Vitro Manipulation", In: *Corn and Corn Improvement,* 3rd edition, Sprague, G.F., et al, (eds.), Agronomy Soc. Amer., pp. 345–387 (1988).

R. L. Phillips, et al., "Elevated Protein–Bound Methionine in Seeds of a Maize Line Resistant to Lysine Plus Threonine", *Cereal Chem, 62,* 213–218 (1985).

J. Poehlman, "Breeding Corn (Maize)", In: *Breeding Field Crops,* 3rd edition, AVI Publishing Co., Westport, CN, pp. 469–471, 477–481 (1986).

Potrykus, I., "Gene Transfer to Cereals: An Assessment," *Bio/Technol., 8,* 535–542 (Jun. 1990).

Potrykus, I., "Gene Transfer to Cereals: An Assessment," *Trends Biotechnol., 7,* 269–273 (Oct. 1989).

I. Potrykus, et al., "Callus formation from stem protoplasts of corn (*Zea mays* L.)", *Mol. Gen. Genet., 156,* 347–350 (1977).

L. M. Prioli, et al., "Plant Regeneration and Recovery of Fertile Plants from Protoplasts of Maize (*Zea mays* L)", *Bio/Technol., 7,* 589–594 (Jun. 1989).

Rhodes, C. A., et al., "Genetically Transformed Maize Plants from Protoplasts," *Science, 240,* 204–207 (Apr. 8, (1988).

Rhodes, C. A., et al., "Plant Regeneration from Protoplasts Isolated from Embryogenic Maize Cell Cultures," *Bio/Technol., 6,* 56–60 (Jan. 1988).

C. A. Rhodes, "Corn: From Protoplasts to Fertile Plants", *Bio/Technol., 7,* 548 (Jun. 1989).

C. A. Rhodes, et al., "Plant Regeneration from Protoplasts Isolated from Embryogenic Maize Cell Cultures", *Bio/Technol., 6,* 56–60 (Jan. 1988).

F. Richard, et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli* dapA Gene", *Biol. Abstracts, 82,* p. AB–391, Abstract No. 3396 (1986).

F. Richard, et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli* dapA Gene", *J. Bacteriol., 166,* 297–300 (1986).

M. C. Ross, et al., "Transient and Stable Transgenic Cells and Calli of Tobacco and Maize Following Microprojectile Bombardment", *J. Cell. Biochem., 13D,* Abstract No. M149, p. 268 (1989).

Sanford, J. C., et al., "Delivery of Substances into Cells and Tissues Using a Particle Bombardment Process", *Particulate Sci. Technol., 5,* 27–37 (1987).

J. C. Sanford, et al., "Attempted Pollen–Mediated Plant Transformation Employing Genomic Donor DNA", *Theor. Appl. Genet., 69,* 571–574 (1985).

J. C. Sanford, "Biolistic Plant Transformation", *Physiol. Plant., 79,* 206–209 (1990).

J. C. Sanford, "The Biolistic Process", *Trends Biotechnol., 6,* 299–302 (1988).

Sanford, J. C., et al., "Attempted Pollen–Mediated Plant Transformation Employing Genomic Donor DNA," *Theor. Appl. Genet., 69,* 571–574 (1985).

A. Schmidt, et al., "Media and environmental effects of phenolics production from tobacco cell cultures", *Chem. Abstracts., 110,* Abstract No. 230156z, p. 514 (1989).

R. D. Schillito, et al., "Regeneration of Fertile Plants From Protoplasts of Elite Inbred Maize", *Bio/Technol., 7,* 581–587 (Jun. 1989).

K. Shimamoto, et al., "Fertile Transgenic Rice Plants Regenerated from Transformed Protoplasts", *Nature, 338,* 274–278 (1989).

R. Smith, et al., "Shoot apex explant for transformation", *Plant Physiology (Suppl.) 86,* Abstract No. 646, p. 108 (1988).

Spencer, et al., "Bialaphos Selection of Stable Transformations from Maize Cell Culture", *Theor. Appl. Genet., 79,* 625–631 (May 1990).

T. M. Spencer, et al., "Selection of Stable Transformants from Maize Suspension Cultures using the Herbicide Bialaphos", Poster presentation, FASEB Plant Gene Expression Conference, Copper Mountain, Colorado (Aug. 8, 1989).

Sprague, et al., "Corn Breeding", In: *Corn and Corn Improvement,* Sprauge, G. F. (ed.), American Society of Agronomy, Inc, Madison, WI, pp. 305, 320–323 (1977).

C. Thompson, et al., "Characterization of the Herbicide–Resistance Gene bar from *Streptomyces hygroscopicus", EMBO J., 6,* 2519–2523 (1987).

D. T. Thomes, et al., "Transgenic Tobacco Plants and their Progeny Derived by Microprojectile Bombardment of Tobacco Leaves", *Plant Mol. Biol., 14,* 261–268 (Feb. 1990).

D. Twell, et al., "Transient Expression of chimeric genes delivered into Pollen by Microprojectile Bombardment", *Plant Physiol., 91,* 1271–1274 (1989).

E. Ulian, et al., "Transformation of Plants via the Shoot Apex", *In Vitro Cell Dev. Biol., 9,* 951–954 (1988).

V. Vasil, et al., "Plant Regeneration from Friable Embryonic Callus and Cell Suspension Cultures of *Zea mays* L", *J. Plant Physiol., 124,* 399–408 (1986).

V. Walbot, et al., "Molecular genetics of corn", In: *Corn and Corn Improvement,* 3rd edition, Sprague, G.F., et al,(eds), American Soc. Agronomy, Madison, WI, pp. 389–430 (1988).

C. Waldron, et al., "Resistance to Hygromycin B", *Plant Mol. Biol., 5,* 103–108 (1985).

Y. Wang, et al., "Transient Expression of Foreign Genes in Rice, Wheat, and Soybean Cells following particle bombardment", *Plant Mol. Biol., 11,* 433–439 (1988).

Weising, K., et al., "Foreign Genes in Plants: Transfer, Structure, Expression and Applications," *Ann. Rev. Genet., 22,* 421–478 (1988).

J. White, et al., "A Cassette Containing the bar Gene of *Streptomyces hygroscopicus:* a Selectable Marker for Plant Transformation", *Nuc. Acid. Res., 18,* 1062 (1989).

Botterman, J., et al., "Engineering Herbicide Resistance in Plants", *Trends in Genet., 4,* 221–222 (Aug. 1988).

Datta, S.K., et al., "Isolated Microspore–Derived Plant Formation via Embryogenesis in *Triticum aestivum* L.", *Plant Sci., 48,* 49–54 (1987).

Hooykaas–Van Slogteren, G.M.S., et al., "Expression of Ti Plasmid Genes in Monocotyledonous Plants Infected with *Agrobacterium tumefaciens", Nature, 311,* 763–764 (Oct. 25, 1984).

Ranch, J.P., et al., "Expression of 5–Methyltryptophan Resistance in Plants Regenerated from Resistant Cell Lines of *Datura innoxia", Plant Physiol., 71,* 136–140 (1983).

Whiteley, H.R., et al., "The Molecular Biology of Parasporal Crystal Body Formation in *Bacillus thuringiensis," Ann. Rev. Microbiol., 40,* 549–576 (1986).

* cited by examiner

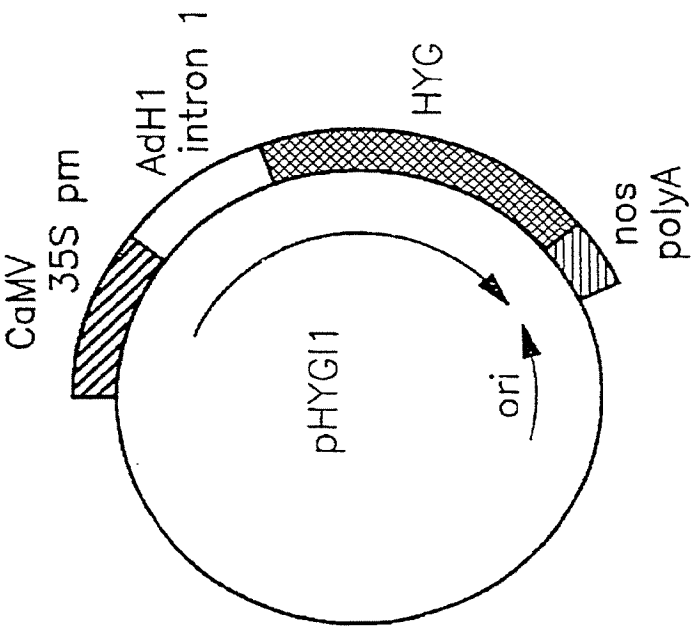
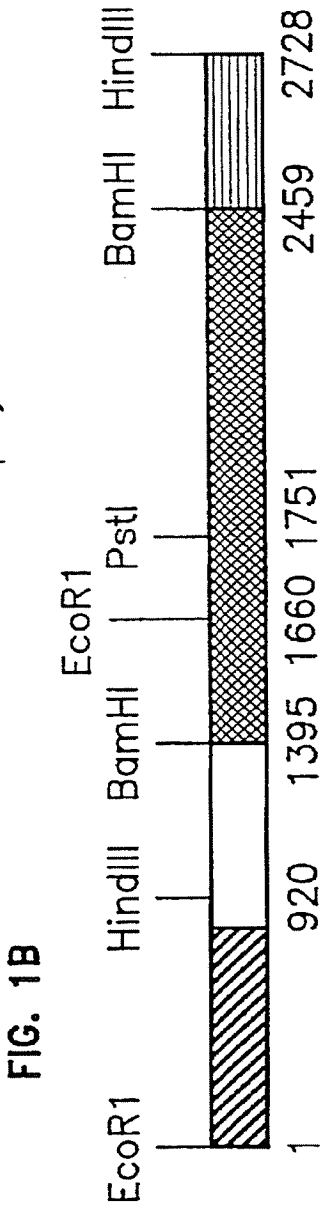

CaMV
35S pm
AdH1
intron 1
HYG
nos
polyA
pHYGI1
ori

FIG. 1A


EcoR1
HindIII
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PstI
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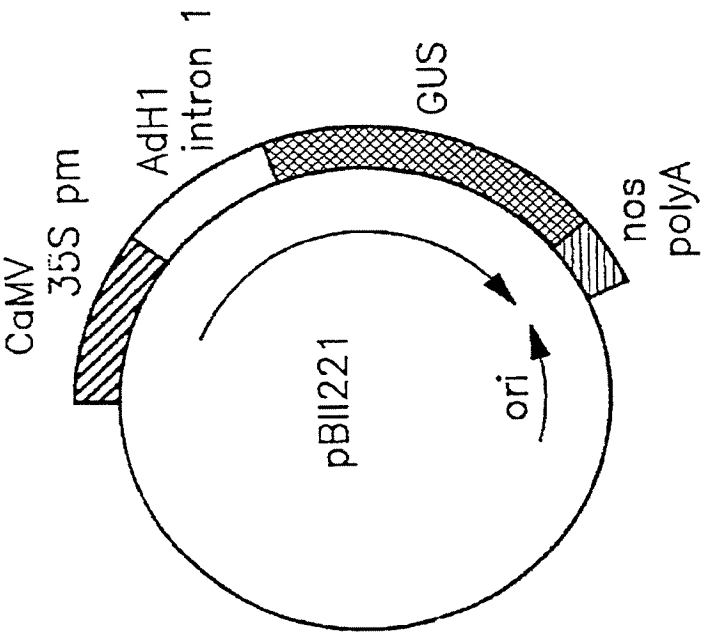
HindIII
BamHI
1
920
1395
1660
1751
2459
2728

FIG. 1B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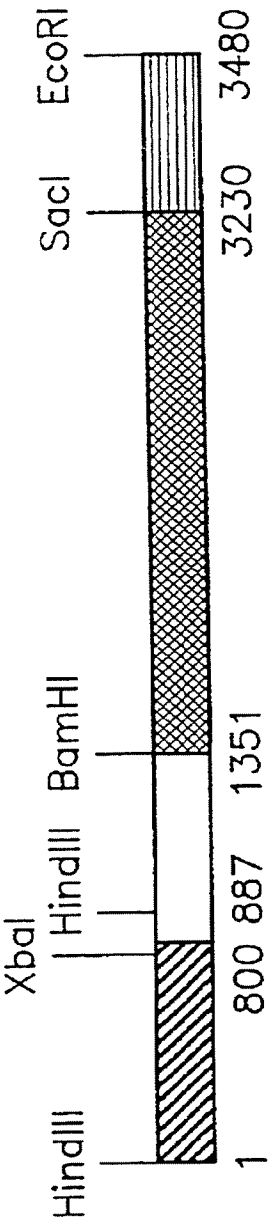
AdH1
intron 1
GUS
nos
polyA
pBII221
ori

FIG. 2A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XbaI
HindIII
887
800
HindIII
1

FIG. 2B

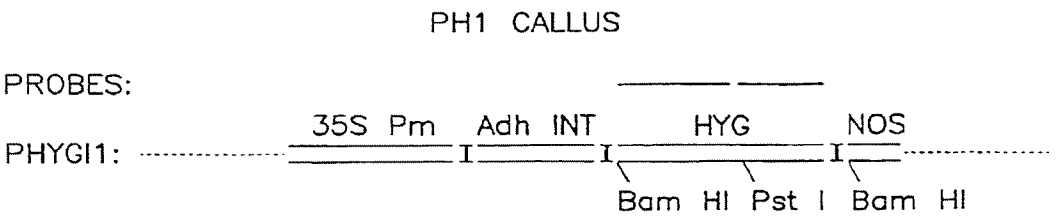

PH1 CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 3A

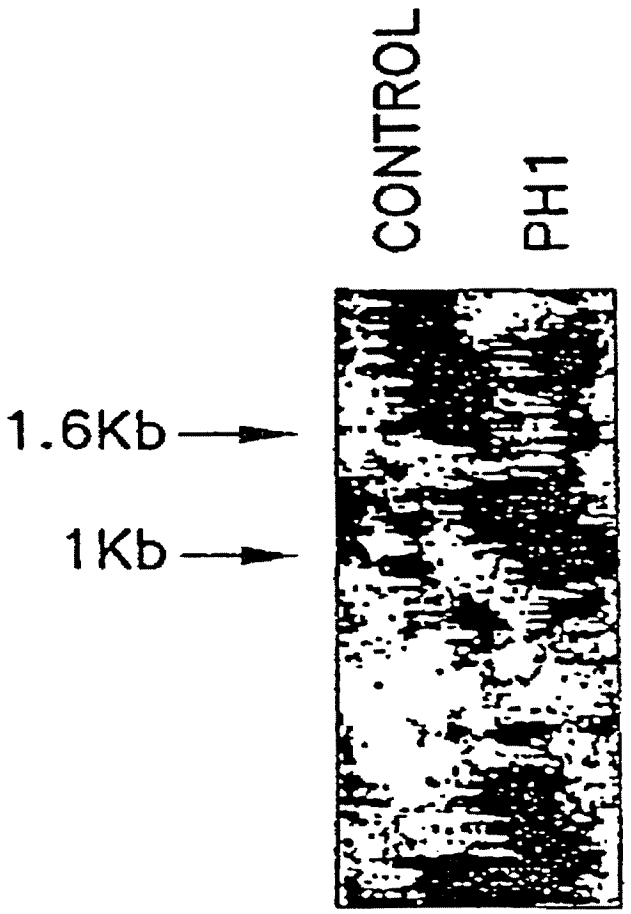


FIG. 3B

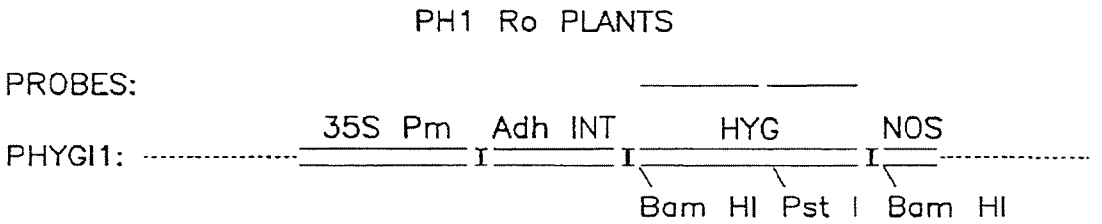

PH1 Ro PLANTS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 4A

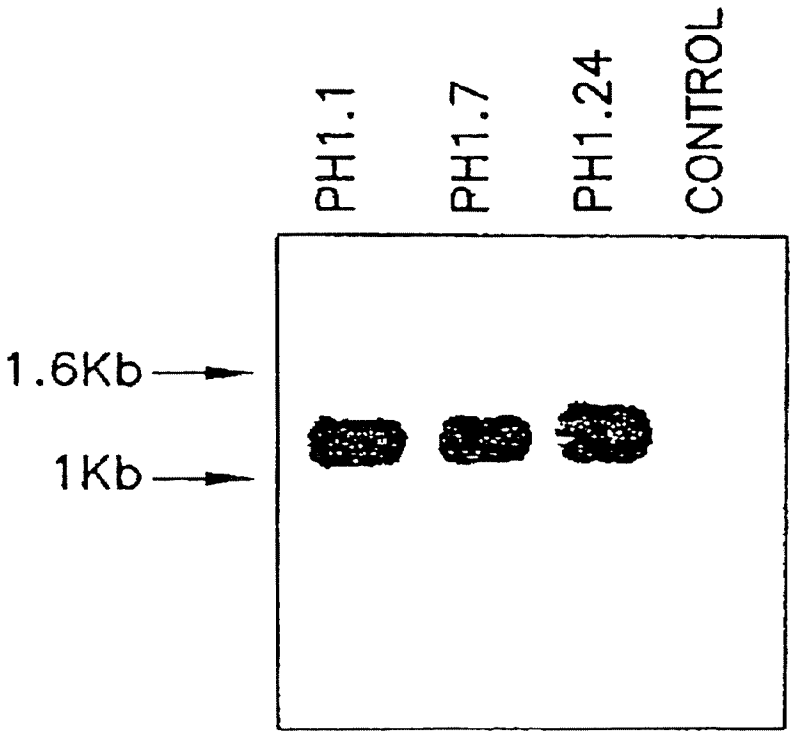
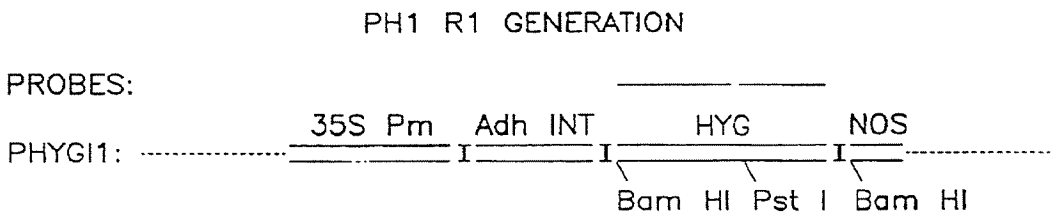


FIG. 4B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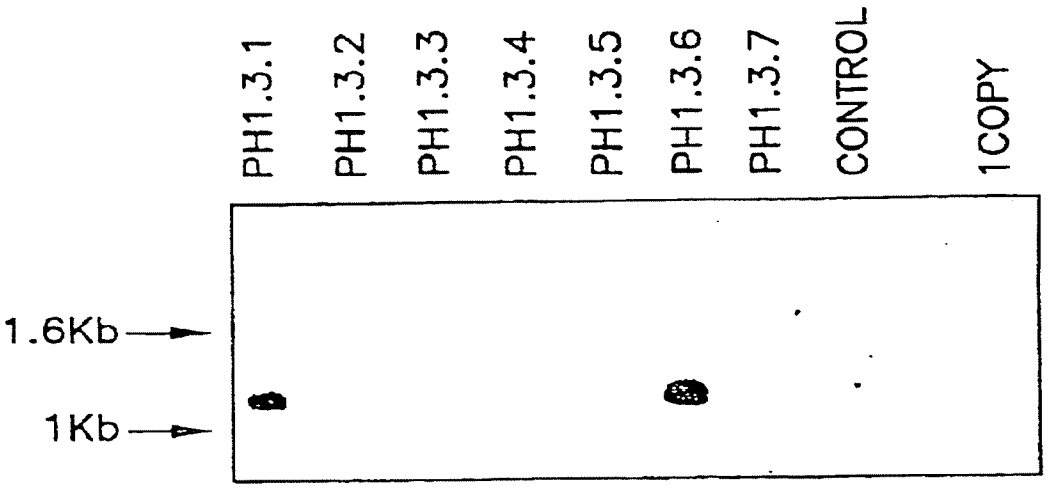
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 5A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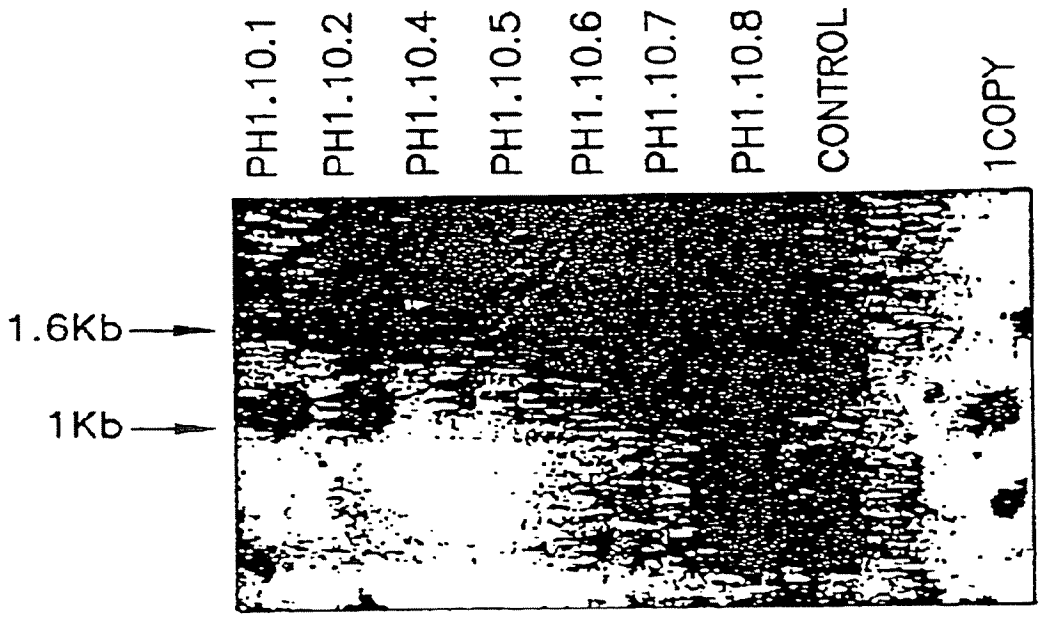
PH1.3.2
PH1.3.3
PH1.3.4
PH1.3.5
PH1.3.6
PH1.3.7
CONTROL
1COPY
1.6Kb
1Kb

FIG. 5B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PH1.10.8
CONTROL
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1.6Kb
1Kb

FIG. 5C

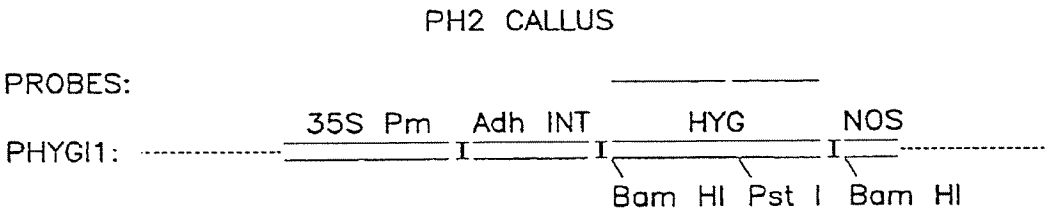
PH2 CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 6A

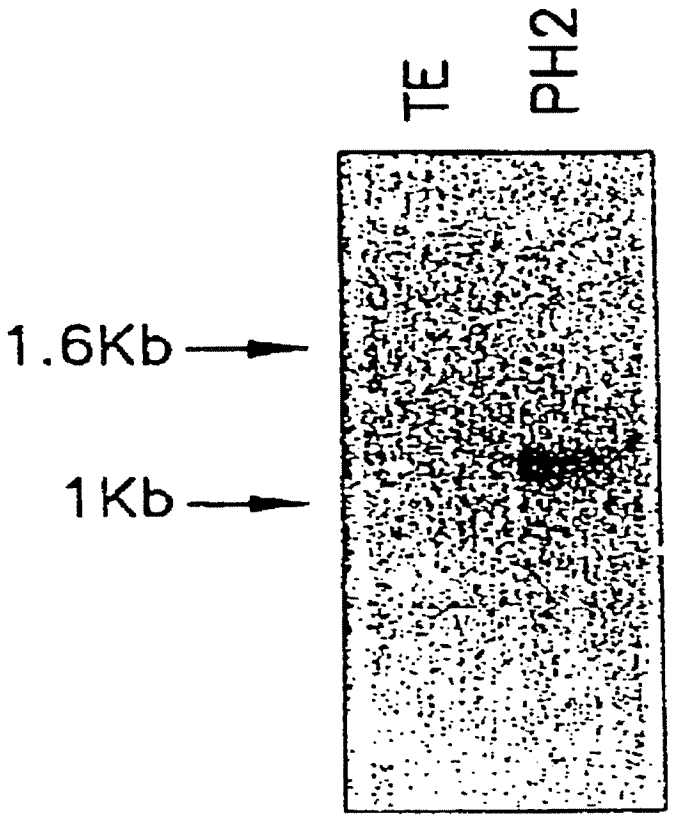

FIG. 6B

METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS

This is a continuation of application Ser. No. 07/974,379, filed Nov. 10, 1992, now U.S. Pat. No. 5,538,877, which application was a continuation of Ser. No. 07/467,983, filed Jan. 22, 1990, now abandoned.

BACKGROUND OF THE INVENTION

This invention relates to fertile transgenic plants of the species *Zea mays* (oftentimes referred to herein as maize or corn). The invention further relates to producing transgenic plants via particle bombardment and subsequent selection techniques which have been found to produce fertile transgenic plants.

Genetic engineering of plants, which entails the isolation and manipulation of genetic material (usually in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells, offers considerable promise to modern agriculture and plant breeding. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals, chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques. Once a gene has been identified, cloned, and engineered, it is still necessary to introduce it into a plant of interest in such a manner that the resulting plant is both fertile and capable of passing the gene on to its progeny.

A variety of methods have been developed and are currently available for the transformation of various plants and plant cells with DNA. Generally these plants have been dicotyledonous, and some success has been reported with certain of the monocotyledonous cereals. However, some species have heretofore proven untransformable by any method. Thus, previous to this discovery, no technology had been developed which would permit the production of stably transformed *Zea mays*' plants in which the transforming DNA is heritable thereof. This failure in the art is well documented in the literature and has been discussed in a number of recent reviews (Potrykus, 1989; Weising et al., 1988; Cocking et al., 1987).

European Patent Publns. 270,356 (McCabe et al.) and 275,069 (Arntzen et al.) describe the introduction of DNA into maize pollen followed by pollination of maize ears and formation of seeds. The plants germinated from these seeds are alleged to contain the introduced DNA, but there is no suggestion that the introduced DNA was heritable, as has been accomplished in the present invention. Only if the DNA introduced into the corn is heritable can the corn be used in breeding programs as required for successful commercialization of transgenic corn.

Graves et al. (1986) claims Agrobacterium-mediated transformation of *Zea mays* seedlings. The alleged evidence was based upon assays known to produce incorrect results.

Despite extensive efforts to produce fertile transformed corn plants which transmit the transforming DNA to progeny, there have been no reported successes. Many previous failures have been based upon gene transfer to maize protoplasts, oftentimes derived from callus, liquid suspension culture cells, or other maize cells using a variety of transformation techniques. Although several of the techniques have resulted in successful transformation of corn cells, the resulting cells either could not be regenerated into corn plants or the corn plants produced were sterile (Rhodes et al. 1988). Thus, while maize protoplasts and some other cells have previously been transformed, the resulting transformants could not be regenerated into fertile transgenic plants.

On the other hand, it has been known that at least certain corn callus can be regenerated to form mature plants in a rather straightforward fashion and that the resulting plants were often fertile. However, no stable transformation of maize callus was ever achieved, i.e. there were no techniques developed which would permit a successful stable transformation of a regenerable callus. An example of a maize callus transformation technique which has been tried is the use of Agrobacterium mediated transfer.

The art was thus faced with a dilemma. While it was known that corn protoplast and suspension culture cells could be transformed, no techniques were available which would regenerate the transformed protoplast into a fertile plant. While it was known that corn callus could be regenerated into a fertile plant, there were no techniques known which could transform the callus, particularly while not destroying the ability of the callus both to regenerate and to form fertile plants.

Recently, a new transformation technique has been created based upon the bombardment of intact cells and tissues with DNA-coated microprojectiles. The technique, disclosed in Sanford et al. (1987) as well as in EPO Patent Publication 331,855 of J. C. Sanford et al. based upon U.S. Ser. No. 161,807, filed Feb. 29, 1988, has been shown effective at producing transient gene expression in some plant cells and tissues including those from onion, maize (Klein et al. 1988a), tobacco, rice, wheat, and soybean, and stable expression has been obtained in tobacco and soybeans. In fact, stable expression has been obtained by bombardment of suspension cultures of *Zea mays* Black Mexican Sweet (Klein et al. 1989) which cultures are, however, non-regenerable suspension culture cells, not the, callus culture cells used in the process of the present invention.

No protocols have been published describing the introduction of DNA by a bombardment technique into cultures of regenerable maize cells of any type. No stable expression of a gene has been reported by means of bombardment of corn callus followed by regeneration of fertile plants and no regenerable fertile corn has resulted from DNA-coated microprojectile bombardment of the suspension cultures. Thus, the art has failed to produce fertile transformed corn plants heretofore.

A further stumbling block to the successful production of fertile transgenic maize plants has been in selecting those few transformants in such a manner that neither the regeneration capacity nor the fertility of the regenerated transformant are destroyed. Due to the generally low level of transformants produced by a transformation technique, the need for selection of the transformants is self-evident. However, selection generally entails the use of some toxic agent, e.g. herbicide or antibiotic, which can effect either the regenerability or the resultant plant fertility.

It is thus an object of the present invention to produce fertile, stably transgenic, *Zea mays* plants and seeds which transmit the introduced gene to progeny. It is a further object to produce such stably transgenic plants and seeds by a particle bombardment and selection process which results in a high level of viability for a few transformed cells. It is a further object to produce fertile stably transgenic plants of other graminaceous cereals besides maize.

REFERENCES CITED

Armstrong, C L, et al. (1985) J Planta 164:207–214
Callis, J, et al. (1987) Genes & Develop 1:1183–1200

M. Bevan et al., *Nuc. Acids Res.*, 11., 369 (1983)
Chu, C C, et al. (1975) Sci Sin (Peking) 18:659–668
Cocking, F, et al; (1987) Science 236:1259–1262
DeWet et al. (1985) Proc Natl Sci USA 82:7870–7873
Freeling, J C, et al. (1976) Maydica XXI:97–112
Graves, A, et al. (1986) Plant Mol Biol 7:43–50
Green, C, et al. (1975) Crop Sci 15:417–421
Green, C E, (1982) Plant Tissue Culture, A Fujiwara ed. Maruzen, Tokyo, Japan pp 107–8
Green, C, et al. (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 367–372
Gritz, L, et al. (1983) Gene 25:179–188
Guilley, H, et al. (1982) Cell 30:763–773
Jefferson, R, et al. (1987) EMBO J. 6:3901–3907)
Kamo, K, et al. (1985) Bot Gaz 146:327–334
Klein, T, et al. (1989) Plant Physiol 91:440–444
Klein, T, et al. (1988a) Proc Natl Acad Sci USA 85:4305–9
Klein, T, et al. (1988b) Bio/Technology 6:559–563
Lu, C, et al. (1982) Theor Appl Genet 62:109–112
McCabe, D, et al. (1988) Bio/Technology 6:923–926
Murashige, T, et al. (1962) Physiol Plant 15:473–497
Neuffer, M, (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 19–30
Phillips, R. et al. (1988) Corn and Corn Improvement, 3rd ed., Agronomy Soc Amer, pp 345–387
Potrykus, I (1989) Trends in Biotechnology 7:269–273
Rhodes, C A, et al. (1988) Science 240:204–7
Sambrook, J, et al (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press
Sanford, J, et al. (1987) J Part Sci & Techn 5:27–37
Weising, K, et al., (1988) Ann Rev of Genetics 22:421–478
Yanisch-Perron, L, et al. (1985) Gene 33:109–119

SUMMARY OF THE INVENTION

The present invention relates to fertile transgenic *Zea mays* plants containing heterologous DNA, preferably chromosomally integrated heterologous DNA, which is heritable by progeny thereof.

The invention further relates to all products derived from transgenic *Zea mays* plants, plant cells, plant parts, and seeds.

The invention further relates to transgenic *Zea mays* seeds stably containing heterologous DNA and progeny which inherit the heterologous DNA.

The invention further relates to a process for producing fertile transgenic *Zea mays* plants containing heterologous DNA. The process is based upon microprojectile bombardment, selection, and plant regeneration techniques.

The invention further relates to a process for producing fertile transformed plants of graminaceous plants other than *Zea mays* which have not been reliably transformed by traditional methods such as electroporation, Agrobacterium, injection, and previous ballistic techniques.

The invention further relates to regenerated fertile mature maize plants from transformed embryogenic tissue, transgenic seeds produced therefrom, and R1 and subsequent generations.

In preferred embodiments, this invention produces the fertile transgenic plants by means of a DNA-coated microprojectile bombardment of clumps of friable embryogenic callus, followed by a controlled regimen for selection of the transformed callus lines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**A shows a map of plasmid vector pHYGI1 utilized in Example I. FIG. **1**B shows the relevant part of pHYGI1

encompassing the HPT coding sequence and associated regulatory elements. The base pair numbers start from the 5' nucleotide in the recognition sequence for the indicated restriction enzymes, beginning with the EcoRI site at the 5' end of the CaMV 35S promoter.

FIG. **2**A shows a map of plasmid vector pBII221 utilized in Example I. FIG. **2**B shows the relevant part of pbII221 encompassing the GUS coding sequence and associated regulatory elements.

FIG. **3**A depicts the pHYG1-containing fragments employed as probes in a Southern blot and analysis of PH1 callus. FIG. **3**B is a Southern blot of DNA isolated from the PH1 callus line and an untransformed control callus line.

FIG. **4**A depicts the pHYG1-containing fragments employed as probes in Southern blot analysis of PH1 Ro plants. FIG. **4**B is a Southern blot of leaf DNA isolated from Ro plants regenerated from PH1 and untransformed callus.

FIG. **5**A depicts the pHYG1- containing fragments employed as probes in Southern blot analysis of PH1 R1 plants. FIG. **5**B and FIG. **5**C are a Southern blot of leaf DNA isolated from R1 progeny of PH1 Ro plants and untransformed Ro plants.

FIG. **6**A depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PH2 callus. FIG. **6**B is a Southern blot of DNA isolated from the PH2 callus line and an untransformed control callus line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the production of fertile transgenic plants and seeds of the species *Zea mays* and to the plants, plant tissues, and seeds derived from such transgenic plants, as well as the subsequent progeny and products derived therefrom. The transgenic plants produced herein include all plants of this species, including field corn, popcorn, sweet corn, flint corn and dent corn.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant which contains heterologous DNA that was introduced into plant material by a process of genetic engineering, or which was initially introduced into a plant species by such a process and was subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions.

By "heritable" is meant that the DNA is capable of transmission through a complete sexual cycle of a plant, i.e. passed from one plant through its gametes to its progeny plants in the same manner as occurs in normal corn.

The transgenic plants of this invention may be produced by (i) establishing friable embryogenic callus from the plant to be transformed, (ii) transforming said cell line by a microprojectile bombardment technique, (iii) controllably identifying or selecting transformed cells, and (iv) regenerating fertile transgenic plants from the transformed cells. Some of the plants of this invention may be produced from the transgenic seed produced from the fertile transgenic plants using conventional crossbreeding techniques to develop commercial hybrid seed containing heterologous DNA.

I. Plant Lines and Tissue Cultures

The cells which have been found useful to produce the fertile transgenic maize plants herein are those callus cells which are regenerable, both before and after undergoing a selection regimen as detailed further below. Generally, these cells will be derived from meristematic tissue which contain cells which have not yet terminally differentiated. Such

tissue in graminaceous cereals in general and in maize, in particular, comprise tissues found in juvenile leaf basal regions, immature tassels, immature embryos, and coleoptilar nodes. Preferably, immature embryos are used. Methods of preparing and maintaining callus from such tissue and plant types are well known in the art and details on so doing are available in the literature, c.f. Phillips et al. (1988), the disclosure of which is hereby incorporated by reference.

The specific callus used must be able to regenerate into a fertile plant. The specific regeneration capacity of particular callus is important to the success of the bombardment/selection process used herein because during and following selection, regeneration capacity may decrease significantly. It is therefore important to start with cultures that have as high a degree of regeneration capacity as possible. Callus which is more than about 3 months and up to about 36 months of age has been found to have a sufficiently high level of regenerability and thus is currently preferred. The regenerative capacity of a particular culture may be readily determined by transferring samples thereof to regeneration medium and monitoring the formation of shoots, roots, and plantlets. The relative number of plantlets arising per Petri dish or per gram fresh weight of tissue may be used as a rough quantitative estimate of regeneration capacity. Generally, a culture which will produce at least one plant per gram of callus tissue will be preferred.

While maize callus cultures can be initiated from a number of different plant tissues, the cultures useful herein are preferably derived from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1–3 mm in length. This length generally occurs about 9–14 days after pollination. Under aseptic conditions, the embryos are placed on conventional solid media with the embryo axis down (scutellum up). Callus tissue appears from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" is meant that the tissue is easily dispersed without causing injury to the cells. Tissue with this morphology is then transferred to fresh media and subcultured on a routine basis about every two weeks.

The callus initiation media is solid because callus cannot be readily initiated in liquid medium. The initiation/maintenance media is typically based on the N6 salts of Chu et al. (1975) as described in Armstrong et al. (1985) or the MS salts of Murashige et al. (1962). The basal medium is supplemented with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth. The cultures are generally maintained in the dark, though low light levels may also be used. The level of synthetic hormone 2,4-D, necessary for maintenance and propagation, should be generally about 0.3 to 3.0 mg/l.

Although successful transformation and regeneration has been accomplished herein with friable embryogenic callus, this is not meant to imply that other transformable regenerable cells, tissue, or organs cannot be employed to produce the fertile transgenic plants of this invention. The only actual requirement for the cells which are transformed is that after transformation they must be capable of regeneration of a plant containing the heterologous DNA following the particular selection or screening procedure actually used.

II. DNA Used for Transformation

The heterologous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of a plasmid and contains coding regions of beneficial heterologous DNA with flanking regulatory sequences which serve to promote the expression of the heterologous DNA present in the resultant corn plant. "Heterologous DNA" is used herein to include all synthetically engineered or biologically derived DNA which is introduced into a plant by man by genetic engineering, including but not limited to, non-plant genes, modified genes, synthetic genes, portions of genes, as well as DNA and genes from maize and other plant species.

The compositions of and methods for constructing heterologous DNA for successful transformations of plants is well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the heterologous DNA useful herein. The specific composition of the DNA is not central to the present invention and the invention is not dependent upon the composition of the specific transforming DNA used. Weising et al. (1988), the subject matter of which is incorporated herein by reference, describes suitable DNA components thereof which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, as well as provides suitable references for compositions thereof. Sambrook et al. (1989) provides suitable methods of construction.

Generally the heterologous DNA will be relatively small, i.e. less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the DNA increases.

Suitable heterologous DNA for use herein includes all DNA which will provide for, or enhance, a beneficial feature of the resultant transgenic corn plant. For example, the DNA may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. For example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; bacterial ESPS synthase for resistance to glyphosate herbicide; chitinase or glucan endo-1,3-B-glucosidase for fungicidal properties. Also, the DNA may be introduced to act as a genetic tool to generate mutants and/or assist in the identification, genetic tagging, or isolation of segments of corn DNA. Additional examples may be found in Table 1.

TABLE 1

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species		Mode of foreign gene expression	References
Animals					
Drosophila heat shock gene hsp 70	5' hsp70/nptII/3' ocs	tobacco (plants, tumors)	+	temperature-dependent and organ-specific	308, 309
Drosophila copia element LTR	5' copia/cat	rice, wheat and sorghum (protoplasts)	(–)	transient, but strong	245

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression	References
firefly luciferase gene	5' CaMV 35S/luciferase cDNA/3' nos 5' CaMV 19S/luciferase cDNA/3' nos 5' deletion series	tobacco (plants) carrot (protoplasts)	– (+) transient	246, 247
Rabbit β-globin gene	genomic	tobacco (tumors)	(–) not expressed	293
Human α-globin gene	5' nos/α-globin	tobacco (plants)	(+) incorrect transcript processing	355
Chicken α-actin gene	genomic	tobacco (tumors)	(–) not expressed	189
Chicken ovalburnin gene	genomic	tobacco (tumors)	(+) incorrect transcript processing	189
Mouse metallothionein gene (mmt)	5' mmt/cat	tobacco (tumors)	(–) not expressed	9
Mouse dihydrofolate reductase gene (DHFR)	5' CaMV 35S/DHFR-cDNA/3' nos	Petunia (plants)	+ expression confers methotrexate resistance	96
Human growth hormone gene (hgh)	5' CaMV 35S/hgh	tobacco (plants, tumors)	(+) incorrect transcript processing	239
	5' nos/hgh/3' nos	tobacco and sunflower (tumors)	(+) transcription, but neither processing nor translation	20
	5' CaMV 35S/hgh/hgh 3'	tobacco (plants)	(+) incorrect transcript polyadenylation	167
SV40 early genes	5' SV40/cat	tobacco (tumors)	(–) not expressed	9
	5' CaMV 35S/cat/3' SV40	tobacco (plants)	(+) incorrect transcript polyadenylation	167
HSV thymidine kinase gene (tk)	5' HSV/tk/cat	tobacco (tumors)	(–) not expressed	9
Adenovirus type 5 EIA gene	5' CaMV 35S/EIA/3' EIA/3' rbcS	tobacco (plants)	(+) termination within rbcS, EIA polyadenylation site not used	167
Yeast				
Yeast ADH	genomic	tobacco (plants)	(–) not expressed	21
Plant virus				
cDNA encoding TMV coat protein (tobacco mosaic virus)	5' CaMV 35S/TMVcDNA/3' nos	tobacco (plants)	+ expression confers enhanced resistance to TMV infection	31, 236, 263
cDNA encoding AMV coat protein (alfalfa mosaic virus)	5' CaMV 19S/AMVcDNA 5' CaMV 35S/AMVcDNA/3' nos	tobacco and tomato (plants)	+ expression confers enhanced resistance to AMV infection	211, 342
cDNAs encoding A- and B-component of TGMV (tomato golden mosaic virus)	separate transformation with either A- or B-component via agroinfection	Petunia (plants)	+ only A-components in tandem are able to replicate	274, 326
cDNA encoding CMV (cucumber mosaic virus) satellite RNA	5' CaMV 35S/CMVcDNA/3' nos	tobacco (plants)	+ expression confers enhanced resistance to CMV infection	23
Plants				
Bean phaseolin gene	5' ocs/phaseolin genomic	sunflower (tumors)	+ expressed and processed correctly	230
	genomic	tobacco (plants)	+ development-specific expression in seeds: targeting to protein bodies in endosperm and embryos	131, 289
	5' phaseolin/phaseolin-cDNA/3' phaseolin	tobacco (tumors)	+ higher expression than using a genomic clone	49
	5' phaseolin/maize zein/3' phaseolin	tobacco (plants)	+ development-specific zein gene expression in tobacco seeds. Zein accumulation	158
Bean phytohemagglutinin-L gene (PHA-L)	genomic	tobacco (plants)	+ development-specific expression in tobacco seeds	362
Soybean β-conglycinin gene (α subunit)	genomic 5' deletion series	Petunia (plants)	+ development-specific expression in Petunia seeds depending on 5' sequences	25, 51
	5' CaMV 35S or 19S/conglycinin/3' nos	Petunia (plants)	+ constitutive expression: 35S > 19S; 20 fold clonal variation	204
Soybean β-conglycinin gene (β subunit)	genomic	tobacco and Petunia (plants)	+ development-specific expression in seeds	35

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species		Mode of foreign gene expression	References
Potato patatin gene	5' patatin/cat/3' nos	potato (plants)	+	organ-specific expression in tubers	343
	5' ST-LS.1/patatin/3' patatin	tobacco (plants)	+	light-regulated and organ-specific expression depending on the ST-LS.1 promoter. Correct splicing of patatin mRNA.	275
Maize zein gene	genomic	sunflower (tumors)	(+)	transcription, but no detectable protein	126, 217
	5' phaseolin/zein/3' phaseolin	tobacco (plants)	+	development-specific expression in tobacco seeds	158
Wheat glutenin genes	5' glutenin/cat/3' nos	tobacco (plants)	+	development-specific expression in tobacco seeds	60
Wheat chlorophyll a/b binding protein (cab) gene	genomic	Petunia and tobacco (plants)	+	light-regulated and organ-specific expression in leaves	201
	genomic 5' cab/cat 5' cab/5' CaMV 35S/cat/ 3' rbcS 5' deletion series	tobacco (plants)	+	phytochrome-regulated expression in leaves depending on 5' sequences	233, 234
Pea cab gene	5' cab/nptII 5' cab/5' nos/nptII	tobacco (plants)	+	light-regulated, organ-and cell-specific expression; depending on enhancer/silencer-like 5' sequences. Involvement of phytochrome. Correlation to the presence of chloroplasts.	303, 304, 305
Petunia cab gene	5' cab/ocs 5' cab/nos	Petunia and tobacco (plants)	+	clonal variation of expression (200 fold) independent of copy number and homo-/heterologous host genome	171, 172
Arabidopsis cab gene	5' cab/cat	tobacco (plants)	+	light-regulated and organ-specific expression	10
Pea ribulose 1.5-Bisphosphate carboxylase small subunit gene (rbcS) E9	genomic 5' rbcS/cat 5' deletion series	Petunia (tumors)	+	light-regulated expression dependent on 5' sequences	38, 225
	genomic 5' deletion series	Petunia and tobacco (plants)	+	light-regulated and organ-specific expression dependent on 5' sequences: 25-fold clonal variation	232
Pea rbcS 3.6	5' rbcS/cat/3' nos 5' deletion series	tobacco (tumors)	+	light-regulated expression dependent on enhancer-like 5' sequences	152, 338
	5' rbcS/rbcS transit sequence/nptII	tobacco (tumors and plants)	+	light-regulated expression and targeting of neomycin phosphotransferase into chloroplasts; analysis of signal sequences	200, 285, 349, 370
	5' rbcS/nptII	tobacco (plants)	+	light-regulated, organ- and cell-specific expression. Involvement of a blue-light receptor	304
Pea rbcS 3A, 3C	genomic	Petunia (plants)	+	regulation of transcription by phytochrome- and/or blue-light receptor depending on the developmental state	105

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species		Mode of foreign gene expression	References
Pea rbcS 3A, E9	5' rbcS/5' CaMV 35S/cat 5' deletion series	Petunia and tobacco (plants)	+	light-regulated and organ-specific expression depending on enhancer- and silencer-like 5' sequences	106, 198
Soybean rbcS	5' rbcS/nptII/3' ocs	soybean (tumors)	+	light-regulated expression	100
	5' rbcS/nos	Kalanchoe (tumors)	+	light-regulated expression	295
	5' rbcS/nptII/3' nos	Petunia (plants)	+	light-regulated expession mediated by phytochrome	300
Soybean, pea and Petunia rbcS	5' rbcS/nptII/3' nos	tomato (plants)	+	expression stronger than directed by nos-promoter	218
Nicotiana plumbaginifolia rbcS 8B	5' rbcS/cat	tobacco and Petunia (plants)	+	light-regulated and organ-specific expression; 3-fold clonal variation independent of homo-/heterologous host genome	262, 341
Wheat rbcS	genomic 5' CaMV 35S/rbcS/3' rbcS	tobacco (plants)	(+)	no expression under the control of wheat promoter; CaMV 35S promoter is necessary	182
Potato ST-LS.1 gene	genomic; modified by exon tagging	potato and tobacco (plants)	+	light-regulated and organ-specific expression depending on the presence of chloroplasts and 5' sequences. Clonal variation parallels copy number, but is independent of homo-/heterologous host.	95, 322
	5' ST-LS.1/patatin/3' patatin	tobacco (plants)	+	light-regulated and organ-specific expression depending on the ST-LS.1 promoter sequences	275
Petroselinum and Antirrhinum chalcone synthase genes (chs)	5' chs(A)/nptII/3' chs(P) 5' deletion series	tobacco (plants)	+	UV-B-light-regulated expression dependent on enhancer-like 5' sequences	180
potato proteinase inhibitor II gene (PI II)	genomic 5' PI II/cat/3' T-DNA gene 6b 5' PI II/cat/3'PI II	tobacco (plants)	+	wound-inducible expression depending on 5' and 3' sequences; systemic spreading by transacting factors	276, 337
Soybean heat shock gene hs 6871	genomic 5' deletion series	sunflower (tumors) and tobacco (plants)	+	temperature-regulated expression depending on 5' sequences	24, 284
Soybean heat shock gene Gmhsp 17.SE	genomic 5' deletion series	sunflower (tumors)	+	expression regulated by temperature and presence of cadmium and arsenite depending on 5' sequences	139
Maize heat shock gene hsp70	genomic	Petunia (plants)	+	temperature-regulated expression	272
Maize alcohol dehydrogenase I gene (AdhI)	5' CaMV 35S/cat/AdhI intron/ 3' rbcS	tobacco (plants)	(+)	maize AdhI intron is not removed from the transcript	182
	5' AdhI/cat 5' ocs/5' AdhI/cat 5' CaMV 35S/5' AdhI/cat 5' AdhI deletion series	tobacco (plants) and maize (protoplasts)	(+)	anaerobically inducible cat-expression dependent on 5' AdhI sequences only if additional CaMV- or ocs-promoter/enhancer sequences are present.	97, 98, 164 366

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species		Mode of foreign gene expression	References
Maize sucrose synthase gene (ss)	5' ss/nptII/3' ocs	wheat (protoplasts)	(+)	transient expression	373
	5' ss/nptII	maize (protoplasts)	(+)	transient expression suspension-culture derived but not in leaf-derived protoplasts	177
Soybean β-tubulin gene	genomic	tobacco (plants)	+		138
cowpea trypsin inhibitor gene (CpTI)	5' CaMV 35S/CpTI/3' nos	tobacco (plants)	+	expression enhances resistance to insect pests	153
Petunia EPSP synthase gene	5' CaMV 35S/EPSP/3' nos	Petunia (plants)	+	35S-directed EPSP overproduction confers glyphosate tolerance	291
	5' T7-T-DNA/Petunia EPSP transit sequence/bacterial EPSP (aroA) coding region	tobaoco (plants)	+	expression of bacterial EPSP; targeting into chloroplasts; glyphosate tolerance	84
Soybean leghemoglobin gene lbc3	genomic	tobacco (plants)		n.d.	310
	5' lbc3/cat/3' lbc3	*Lotus corniculatus* (plants)	+	development-specific and inducible expression only in nodules and only after infection by Rhizobium; dependence on 5' regulatory sequences	170, 323, 324
Nicotiana plumbaginifolia "insert 7" enhancer-like sequence	5' insert 7/5' nos/nptII	tobacco (plants)	+	transient protoplast-specific overexpression of nptII	163
Nicotiana plumbaginifolia ATP synthase gene (atp2-1); β subunit	5' CaMV 35S/atp2-1 signal sequence/cat	tobacco (plants)	+	cat enzyme is targeted into mitochondria	34
Maize transposable elements Ac and Ds	Ac or Ds within borders of waxy locus	tobacco (tumors and shoots)	+	Ac is capable of self-catalyzed transposition	15, 16

Abbreviations:
copia LTR - long terminal repeat of copia transposable element
HSV - Herpes simplex virus
Adh - alcohol dehydrogenase gene
ST-LS 1 - Solanum ruberosum leaf/stem-specific gene
EPSP - 5-enolpyruvylshikimate-3-phosphate (gene)
lbc3 - member of the leghemoglobin gene family
Ac, Ds - maize transposable elements (activator, dissociation)
rbcS - ribulose-1,5-bisphosphate carboxylase small subunit gene
cab - chlorophyl a/b binding protein gene
nptII - neomycin phosphotransferase II gene
ocs - octopine synthase gene
nos - nopaline synthase gene
cat - chloramphenicol acetyltransferase gene
CaMV 35S, 19S - cauliflower mosaic virus genes encoding 35S and 19S-transcript, respectively
genomic - transferred construct contains the entire gene including 5' and 3' regions
Mode of expression:
+ - correct expression of stably integrated gene
(+) - transient expression, or transcription followed by incorrect processing and/or translation
(−) - gene is not transcribed

The heterologous DNA to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a cotransformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Table 2.

Selectable marker and reporter genes in plant genetic transformation

Gene	Origin	Encoded enzyme	Useful as: Selectable marker	Useful as: Scorable reporter	Resistance against
Neomycin phosphotransferase gene II (nptII)	Tn5	neomycin phosphotransferase	++	+	neomycin kanamycin G-418[1]
Neomycin phosphotransferase gene I (nptI)	Nn601	neomycin phosphotransferase	+	+	neomycin kanamycin G-418[2]
Chloramphenicol acetyltransferase gene (cat)	Tn9	chloramphenicol acetyltransferase	(+)	++	chloramphenicol[3]
Bacterial DHFR gene	plasmid R67	dihydrofolate reductase	+	+	methotrexate[4]
Mutated c-DNA of a mouse DHFR gene	mouse	dihydrofolate reductase	++	+	methotrexate[5]
Octopine synthase gene (ocs)	T-DNA	octopine synthase	+	++	toxic opine precursor analogues, i.e. aminoethylcystein[6]
Nopaline synthase gene (nos)	T-DNA	nopaline synthase	–	++	—[7]
Hygromycin phosphotransferase gene (hpt)	*E. coli*	hygromycin phosphotransferase	++	–	hygromycin B[8]
Bleomycin resistance gene	Tn5	?	+	–	bleomycin[9]
Streptomycin phosphotransferase gene	Tn5	streptomycin phosphotransferase	(+)	(+)	streptomycin[10]
			control plants are not killed by streptomycin		
aroA gene	*Salmonella typhimurium*	EPSP synthase	++	–	glyphosate[11]
bar gene	*Streptomices hygroscopicus*	phosphinothricin acetyltransferase	++	–	phosphinothricin, bialaphos[12]
β-galactosidase gene	*E. coli*	β-galactosidase	–	+	—[13]
Glucuronidase gene (GUS)	*E. coli*	glucuronidase	–	++	—[14]
Bacterial luciferase gene	*Vibrso harvest*	luciferase	–	++	—[15]
Firefly luciferase gene	*Photinus peralis*	luciferase	–	++	—[16]

Only some representative references were chosen in case of the nptII, nos, ocs and cat genes.
Abbreviations
Tn—transpuson
DHFR—dihydrofolate reductase
EPSP synthase—5-enolpyruvylskikimate-3-phosphate synthase

[1]M. Bevran et al., Nature, 304, 185 (1983); M. DeBlock et al., EMBO J., 8, 1681 (1984); I. Herrera-Estrella et al., EMBO J., 2, 987 (1983).
[2]R. T. Fraley et al., PNAS USA, 80, 1803 (1983); H. Pretrzak et al., Nucl. Acids Res., 14, 5857 (1986).
[3]M. DeBlock et al., EMBO J., 3, 1681 (1984); I. Herrera-Estrella et al., Nature, 303, 209 (1983).
[4]N. Brisson et al., Nature, 310, 511 (1984); M. DeBlock et al., ibid., I. Herrera-Estrella et al., EMBO J., 2, 987 (1983).
[5]D. A. Eichholtz et al., Somat. Cell. Mol. Genet., 13, 67 (1987).
[6]G. A. Dahl et al., Theor. Appl. Genet., 66, 233 (1983); H. De Geve et al., Nature, 300, 752 (1982); A. Hockema et al., Plant Mol. Biol., 5, 85 (1985); M. G. Koztel et al., J. Mol. Appl. Genet., 2, 549 (1981).
[7]J. D. G. Jones et al., EMBO J., 4, 2411 (1985); C. H. Shaw et al., Nuc. Acids Res., 14, 6003 (1986); P. Zambryska et al., EMBO J., 2, 2443 (1983).
[8]A. M. Lloyd et al., Science, 284, 464 (1986); P. I. M. Van den Hazen et al., Plant MOl, Biol., 5, 299 (1985); C. Waldron et al., Plant Mol. Biol., 5, 103 (1985).
[9]J. Hille et al., Plant Mol. Biol., 7, 171 (1986).
[10]J. D. G. Jones et al., Mol. Gen. Genet., 210, 86 (1987).
[11]L. Comai et al., Nature, 317, 741 (1985); J. J. Inllatti et al., Biotechnology, 5, 726 (1987).
[12]M. DeBlock et al., EMBO J., 6, 2513 (1987); C. I. Thompson et al., EMBO J., 6, 2519 (1987).
[13]G. Heimer et al., Biotechnology, 2, 520 (1984).
[14]D. R. Gallie et al., Nuc. Acids Res., 15, 8693 (1987); R. A. Jefferson et al., EMBO J., 6, 1901 (1987).
[15]C. Koncz et al., Mol. Gen. Genet., 204, 383 (1986).
[16]D. W. Ow et al., Science, 234, 856 (1986); D. W. Ow et al., PNAS USA, 84, 4870 (1987); C. D. Riggs et al., Nucl. Acids Res., 15, 8115 (1987).

A preferred selectable marker gene is the hygromycin B phosphotransferase (HPT) coding sequence, which may be derived from *E. coli*. Other selectable markers known in the art include aminoglycoside phosphotransferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin, and G418, as well as those genes which code for resistance or tolerance to glyphosate, methotrexate, imidazolinones, sulfonylureas, bromoxynil, dalapon, and the like. Those selectable marker genes which confer herbicide resistance or tolerance are also of commercial utility in the resulting transformed plants.

Reporter genes which encode for easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g. phenotypic change or enzymatic activity Examples of such genes are provided in Table 2. Preferred genes include the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the beta-glucuronidase gene of the uidA locus of *E. coli*, and the luciferase genes from firefly *Photinus pyralis*.

The regulatory sequences useful herein include any constitutive, inducible, tissue or organ specific, or developmental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al, supra. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *Agrobacterium tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase

promoter from corn; light inducible promoters such as, ribulose-biphosphate-carboxylase small subunit gene from a variety of species; and the major chlorophyll a/b binding protein gene promoter; 35S and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present on the DNA. Such elements may or may not be necessary for the function of the DNA, although they can provide a better expression or functioning of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the plant. For example, the maize AdhlS first intron may be placed between the promoter and the coding sequence of a particular heterologous DNA. This intron, when included in a DNA construction, is known to generally increase expression in maize cells of a protein. (Callis et al. 1987) However, sufficient expression for a selectable marker to perform satisfactorily can often be obtained without an intron. (Klein et al. 1989) An example of an alternative suitable intron is the shrunken-1 first intron of *Zea mays*. These other elements must be compatible with the remainder of the DNA constructions.

To determine whether a particular combination of DNA and recipient plant cells are suitable for use herein, the DNA may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the DNA has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (GUS) gene (Jefferson et al. 1987). In the case of the microprojectile bombardment transformation process of the present invention, a suitable time for conducting the assay is about 2–3 days after bombardment. The use of transient assays is particularly important when using DNA components which have not previously been demonstrated or confirmed as compatible with the desired recipient cells.

III. DNA Delivery Process

The DNA can be introduced into the regenerable maize callus cultures via a particle bombardment process. A general description of a suitable particle bombardment instrument is provided in Sanford et al. (1987), the disclosure of which is incorporated herein by reference. While protocols for the use of the instrument in the bombardment of maize non-regenerable suspension culture cells are described in Klein et al. (1988a, 1988b, and 1989), no protocols have been published for the bombardment of callus cultures or regenerable maize cells.

In a microprojectile bombardment process, also referred to as a biolistic process, the transport of the DNA into the callus is mediated by very small particles of a biologically inert material. When the inert particles are coated with DNA and accelerated to a suitable velocity, one or more of the particles is able to enter into one or more of the cells where the DNA is released from the particle and expressed within the cell. While some of the cells are fatally damaged by the bombardment process, some of the recipient cells do survive, stably retain the introduced DNA, and express it.

The particles, called microprojectiles, are generally of a high density material such as tungsten or gold. They are coated with the DNA of interest. The microprojectiles are then placed onto the surface of a macroprojectile which serves to transfer the motive force from a suitable energy source to the microprojectiles. After the macroprojectile and the microprojectiles are accelerated to the proper velocity, they contact a blocking device which prevents the macroprojectile from continuing its forward path but allows the DNA-coated microprojectiles to continue on and impact the recipient callus cells. Suitable such instruments may use a variety of motive forces such as gunpowder or shock waves from an electric arc discharge (C. Sanford et al., *J. Particle Science and Technology,* 5, 27 (1987)). An instrument in which gunpowder is the motive force is currently preferred and such is described and further explained in Sanford et al. (1987), the disclosure of which is incorporated herein by reference.

A protocol for the use of the gunpowder instrument is provided in Klein et al. (1988a, b) and involves two major steps. First, tungsten microprojectiles are mixed with the DNA, calcium chloride, and spermidine free base in a specified order in an aqueous solution. The concentrations of the various componenets may be varied as taught. The currently preferred procedure entails exactly the procedure of Klein et al. (1988b) except for doubling the stated optimum DNA concentration. Secondly, in the actual bombardment, the distance of the recipient cells from the end of the barrel as well as the vacuum in the sample chamber. The currently preferred procedure for bombarding the callus entails exactly the procedure of Klein et al. (1988b) with the recipient tissue positioned 5 cm below the stopping plate tray.

The callus cultures useful herein for generation of transgenic plants should generally be about 3 months to 3 years old, preferably about 3 to 18 months old. Callus used for bombardment should generally be about midway between transfer periods and thus past any "lag" phase that might be associated with a transfer to a new media, but also before reaching any "stationary" phase associated with a long time on the same plate.

The specific tissue subjected to the bombardment process is preferably taken about 7–10 days after subculture, though this is not believed critical. The tissue should generally be used in the form of pieces of about 30 to 80, preferably about 40 to 60, mg. The clumps are placed on a petri dish or other surface and arranged in essentially any manner, recognizing that (i) the space in the center of the dish will receive the heaviest concentration of metal-DNA particles and the tissue located there is likely to suffer damage during bombardment and (ii) the number of particles reaching a cell will decrease (probably exponentially) with increasing distance of the cell from the center of the blast so that cells far from the center of the dish are not likely to be bombarded and transformed. A mesh screen, preferably of metal, may be laid on the dish to prevent splashing or ejection of the tissue. The tissue may be bombarded one or more times with the DNA-coated metal particles.

IV. Selection Process

Once the calli have been bombarded with the DNA and the DNA has penetrated some of the cells, it is necessary to identify and select those cells which both contain the heterologous DNA and still retain sufficient regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively and preferably, when a selectable marker gene has been transmitted along

with or as part of the heterologous DNA, those cells of the callus which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells while simultaneously inhibiting the growth of the non-transformed cells. The situation is complicated by the fact that the vitality of individual cells in a population is often highly dependent on the vitality of neighboring cells. Also, the selection conditions must not be so severe that the plant regeneration capacity of the callus cells and the fertility of the resulting plant are precluded. Thus the effects of the selection agent on cell viability and morphology should be evaluated. This may be accomplished by experimentally producing a growth inhibition curve for the given selective agent and tissue being transformed beforehand. This will establish the concentration range which will inhibit growth.

When a selectable marker gene has been used, the callus clumps may be either allowed to recover from the bombardment on non-selective media or, preferably, directly transferred to media containing that agent.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably the concentration of the agent is initially such that about a 5–40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells to preferentially grow and divide while inhibiting untransformed cells, but not to the extent that growth of the transformed cells is prevented. Once the few individual transformed cells have grown sufficiently the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of non-transformed cells habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative maize transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells. The callus may also be cultured on non-selective media at various times during the overall selection procedure.

Once a callus sector is identified as a putative transformant, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e. Southern blot or polymerase chain reaction (PCR) or the like.

V. Regeneration of Plants and Production of Seed

Cell lines which have been shown to be transformed must then be regenerated into plants and the fertility of the resultant plants determined. Transformed lines which test positive by genotypic and/or phenotypic analysis are then placed on a media which promotes tissue differentiation and plant regeneration. Regeneration may be carried out in accordance with standard procedures well known in the art. The procedures commonly entail reducing the level of auxin which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. One example of such a regeneration procedure is described in Green et al. (1981). The plants are grown to maturity in a growth room or greenhouse and appropriate sexual crosses and selfs are made as described by Neuffer (1981).

Regeneration, while important to the present invention, may be performed in any conventional manner. If a selectable marker has been transformed into the cells, the selection agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are transformed. Since regeneration techniques are well known and not critical to the present invention, any technique which accomplishes the regeneration and produces fertile plants may be used.

VI. Analysis of R1 Progeny

The plants regenerated from the transformed callus are referred to as the RO generation or RO plants. The seeds produced by various sexual crosses of the RO generation plants are referred to as R1 progeny or the R1 generation. When R1 seeds are germinated, the resulting plants are also referred to as the R1 generation.

To confirm the successful transmission and inheritance of the heterologous DNA in the sexual crosses described above, the R1 generation should be analyzed to confirm the presence of the transforming DNA. The analysis may be performed in any of the manners such as were disclosed above for analyzing the bombarded callus for evidence of transformation, taking into account the fact that plants and plant parts are being used in place of the callus.

VII. Breeding of Genetically Engineered Commercial Hybrid Seed

Generally, the commercial value of the transformed corn produced herein will be greatest if the heterologous DNA can be incorporated into many different hybrid combinations. A farmer typically grows several varieties of hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based upon his physical location since hybrids adapted to one part of the corn belt are generally not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the heterologous DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable heterologous DNA. This may conveniently be done by breeding programs in which a conversion process (backcrossing) is performed by crossing the initial transgenic fertile plant to normal elite inbred lines and then crossing the progeny back to the normal parent. The progeny from this cross will segregate such that some of the plants will carry the heterologous DNA whereas some will not. The plants that do carry the DNA are then crossed again to the normal plant resulting in progeny which segregate once more. This crossing is repeated until the original normal

parent has been converted to a genetically engineered line containing the heterologous DNA and also possessing all other important attributes originally found in the parent. A separate backcrossing program will be used for every elite line that is to be converted to a genetically engineered elite line. It may be necessary for both parents of a hybrid seed corn to be homozygous for the heterologous DNA. Corn breeding and the techniques and skills required to transfer genes from one line or variety to another are well-known to those skilled in the art. Thus introducing heterologous DNA into lines or varieties which do not generate the appropriate calli can be readily accomplished by these breeding procedures.

VIII. Uses of Transgenic Plants

The transgenic plants produced herein are expected to be useful for a variety of commercial and research purposes. Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g. agronomic traits such as pest resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g. improved nutritive content in human food or animal feed), or beneficial to the food processor (e.g. improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods however, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silage or for ornamental purposes (e.g. Indian corn). Often chemical constituents (e.g. oils or starches) of corn and other crops are extracted for food or industrial use and transgenic plants may be created which have enhanced or modified levels of such components. The plants may also be used for seed production for a variety of purposes.

Transgenic plants may also find use in the commercial manufacture of proteins or other molecules encoded by the heterologous DNA contained therein, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules, or for other purposes (e.g. for research).

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded by the heterologous DNA may be transferred, e.g. from corn cells to cells of other species e.g. by protoplast fusion.

The transgenic plants may have many uses in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. The methods of the invention may also be used to create plants having unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties.

The following non-limiting examples are illustrative of the present invention. They are presented to better explain the general procedures which were used to prepare the fertile *Zea mays* plants of this invention which stably express the heterologous DNA and which transmit that DNA to progeny. All parts and percents are by weight unless otherwise specified. It must be recognized that a specific transformation event is a function of the amount of material subjected to the transformation procedure. Thus when individual situations arise in which the procedures described herein do not produce a transformed product, repetition of the procedures will be required.

EXAMPLE I

Fertile transgenic *Zea mays* plants which contain heterologous DNA which is heritable were prepared as follows:

I. Initiation and Maintenance of Maize Cell Cultures Which Retain Plant Regeneration Capacity

Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred line A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient agar initiation/maintenance media with the root/shoot axis exposed to the medium. Initiation/maintenance media (hereinafter refered to as F medium) consisted of N6 basal media (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc. San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

The immature embryos were incubated at 26° C. in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh media. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell culture named AB12 used in this example was such a culture and had been initiated about 1 year before bombardment.

II. Plasmids—pCHN1-1, pHYGI1, pBII221, and pLUC-1

The plasmids pCHN1-1, pHYGI1, and pLUC-1 were constructed in the vector pBS+ (Stratagene, Inc., San Diego, Calif.), a 3.2 Kb circular plasmid, using standard recombinant DNA techniques. pCHN1-1 contains the hygromycin B phosphotransferase (HPT) coding sequence from *E. coli* (Gritz et al. 1983) flanked at the 3' end by the nopaline synthase (nos) polyadenylation sequence of *Agrobacterium tumefaciens* (Chilton and Barnes 1983). Expression is driven by the cauliflower mosaic virus (CaMV 35S promoter (Guilley et al. 1982), located upstream from the hygromycin coding sequence. The plasmid pHYGI1 was constructed by inserting the 553 bp Bcl-BamHI fragment containing the maize Adh1S first intron (Callis et al. 1987) between the CaMV 35 S promoter and the hygromycin coding sequence of pCHN1-1. A map of pHYGI1 is provided as FIG. 1A.

pBII221 contains the *E. Coli* β-glucuronidase coding sequence flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. The plasmid was constructed by inserting the maize AdhIS first intron between the 35S promoter and the coding sequence of pBII221 (Jefferson et al. 1987). A map of pBII221 is provided as FIG. 2A.

pLUC-1 contains the firefly luciferase coding sequence (DeWet et al. 1987) flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. This plasmid was used solely as a negative control.

Plasmids were introduced into the embryogenic callus culture AB12 by microprojectile bombardment.

III. DNA Delivery Process

The embryogenic maize callus line AB12 was subcultured 7 to 12 d prior to microprojectile bombardment. AB12 was prepared for bombardment as follows. Five clumps of callus, each approximately 50 mg in wet weight were arranged in a cross pattern in the center of a sterile 60×15 mm petri plate (Falcon 1007). Plates were stored in a closed container with moist paper towels throughout the bombardment process. Twenty six plates were prepared.

Plasmids were coated onto M-10 tungsten particles (Biolistics) exactly as described by Klein, et al (1988b) except that, (i) twice the recommended quantity of DNA was used, (ii) the DNA precipitation onto the particles was performed at 0° C., and (iii) the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process.

All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5 M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. When two plasmids were used simultaneously, each was present in an amount of 2.5 ul. One tube contained only plasmid pBII221; two tubes contained both plasmids pHYGI1 and pBII221; two tubes contained both plasmids pCHN1-1 and pBII221; and one tube contained only plasmid pLUC-1.

All tubes were incubated on ice for 10 min., pelletized by centrifugation in an Eppendorf centrifuge at room temperature for 5 seconds, and 25 ul of the supernatant was discarded. The tubes were stored on ice throughout the bombardment process. Each preparation was used for no more than 5 bombardments.

Macroprojectiles and stopping plates were obtained from Biolistics, Inc. (Ithaca, N.Y.). They were sterilized as described by the supplier. The microprojectile bombardment instrument was obtained from Biolistics, Inc.

The sample plate tray was positioned at the position 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate in the slot below the barrel. Plates of callus tissue prepared as described above were centered on the sample plate tray and the petri dish lid removed. A 7×7 cm square rigid wire mesh with 3×3 mm mesh and made of galvanized steel was placed over the open dish in order to retain the tissue during the bombardment. Tungsten/DNA preparations were sonicated as described by Biolistics, Inc. and 2.5 ul was pipetted onto the top of the macroprojectiles. The instrument was operated as described by the manufacturer. The following bombardments were performed:

2 × pBII221 prep	To determine transient expression frequency
10 × pHYGI1/pBII221	As a potential positive treatment for transformation
10 × pCHN1-1/pBII221	As a potential positive treatment for transformation
4 × pLUC-1	Negative control treatment

The two plates of callus bombarded with pBII221 were transferred plate for plate to F medium (with no hygromycin) and the callus cultured at 26° C. in the dark. After 2 d this callus was then transferred plate for plate into 35×10 mm petri plates (Falcon 1008) containing 2 ml of GUS assay buffer which consists of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (Research Organics), 100 mM sodium phosphate pH 7.0, 5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM EDTA, and 0.06% Triton X-100. These were incubated at 37° C. for 3 d after which the number of blue cells was counted giving 291 and 477 transient GUS expressing cells in the two plates, suggesting that the DNA delivery process had also occurred with the other bombarded plates. These plates were discarded after counting since the GUS assay is destructive.

IV. Selection Process

Hygromycin B (Calbiochem) was incorporated into the medium by addition of the appropriate volume of filter sterilized 100 mg/ml Hygromycin B in water when the media had cooled to 45° C. prior to pouring plates.

Immediately after all samples had been bombarded, callus from all of the plates treated with pHYGI1/pBII221, pCHN1-1/pBII221 and three of the plates treated with pLUC-1 were transferred plate for plate onto F medium containing 15 mg/l hygromycin B, (five pieces of callus per plate). These are referred to as round 1 selection plates. Callus from the fourth plate treated with pLUC-1 was transferred to F medium without hygromycin. This tissue was subcultured every 2–3 weeks onto nonselective medium and is referred to as unselected control callus.

After two weeks of selection, tissue appeared essentially identical on both selective and nonselective media. All callus from eight plates from each of the pHYGI1/pBII221 and pCHN1-1/pBII221 treatments and two plates of the control callus on selective media were transferred from round 1 selection plates to round 2 selection plates that contained 60 mg/l hygromycin. The 2 selection plates each contained ten 30 mg pieces of callus per plate, resulting in an expansion of the total number of plates.

The resulting tissue on selective media, two plates each of pHYGI1/pBII221 and PCMN1-1/pbII221 treated tissue and one of control callus, were placed in GUS assay buffer at 37° C. to determine wheather blue clusters of cells were observable at two weeks post-bombardment. After 6 d in assay buffer this tissue was scored for GUS expression.

TREATMENT	REPLICATE	OBSERVATIONS
pLUC-1		no blue cells
pHYGI1/pBII221	plate 1	11 single cells 1 four cell cluster
	plate 2	5 single cells
pCHN1-1/pBII221	plate 1	1 single cell 2 two cell clusters
	plate 2	5 single cells 1 two cell cluster 2 clusters of 8–10 cells

After 21 d on the round 2 selection plates, all viable portions of the material were transferred to round 3 selection plates containing 60 mg/l hydrogen. The round 2 selection plates, containing only tissue that was apparently dead, were reserved. Both round 2 and 3 selection plates were observed periodically for viable proliferating sectors.

After 35 d on round 3 selection plates both the round 2 and round 3 sets of selection plates were checked for viable sectors of callus. Two such sectors were observed proliferating from a background of dead tissue on plates treated with pHYGI1/pBII221. The first sector named 3AA was from the round 3 group of plates and the second sector named 6L was from the round 2 group of plates. Both lines were then transferred to F medium without hygromycin.

After 19 d on F medium without hygromycin the line 3AA grew very little whereas the line 6L grew rapidly. Both were transferred again to F medium for 9 d. The lines 3AA and 6L

were then transferred to F medium containing 15 mg/l hygromycin for 14 d. At this point, line 3AA was observed to be of very poor quality and slow growing. The line 6L however grew rapidly on F medium with 15 mg.l hygromycin. In preparation for an inhibition study of the line 6L on hygromycin, the line was then subcultured to F medium without hygromycin.

After 10 d on F medium an inhibition study of the line 6L was initiated. Callus of 6L was transfered onto F medium containing 0, 10, 30, 100, and 250 mg/l hygromycin B. Five plates of callus were prepared for each concentration and each plate contained ten approximately 50 mg pieces of callus. One plate of unselected control tissue was prepared for each concentration of hygromycin.

It was found that the line 6L was capable of sustained growth over 9 subcultures on 0, 10, 30, 100, and 250 mg/l hygromycin. The name of the line 6L was changed at this time from 6L to PH1 (Positive Hygromycin transformant 1).

Additional sectors were recovered at various time points from the round 2 and 3 selection plates. None of these were able to grow in the presence of hygromycin for multiple rounds, i.e. two or three subcultures.

V. Confirmation of Transformed Callus

To show that the PH1 callus had acquired the hygromycin resistance gene, a Southern blot of PH1 callus was prepared as follows: DNA was isolated from PH1 and unselected control calli by freezing 2 g of callus in liquid nitrogen and grinding it to a fine powder which was transferred to a 30 ml Oak Ridge tube containing 6 ml extraction buffer (7M urea, 250 mM NaCl, 50:mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1% sarcosine). To this was added 7 ml of phenol:chloroform 1:1, the tubes shaken and incubated at 37° C. 15 min. Samples were centrifuged at 8K for 10 min. at 4° C. The supernatant was pipetted through miracloth (Calbiochem 475855) into a disposable 15 ml tube (American Scientific Products, C3920-15A) containing 1 ml 4.4 M ammonium acetate, pH 5.2. Isopropanol, 6 ml, was added, the tubes shaken, and the samples incubated at −20° C. for 15 min. The DNA was pelleted in a Beckman TJ-6 centrifuge at the maximum speed for 5 min. at 4° C. The supernatant was discarded and the pellet was dissolved in 500 ul TE-10 (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) 15 min. at room temperature. The samples were transferred to a 1.5 ml Eppendorf tube and 100 ul 4.4 M. ammonium acetate, pH 5.2 and 700 ul isopropanol were added. This was incubated at −20° C. for 15 min. and the DNA pelleted 5 min. in an Eppendorf microcentrifuge (12,000 rpm). The pellet was washed with 70% ethanol, dried, and resuspended in TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The isolated DNA (10 ug) was digested with BamHI (NEB) and electrophoresed in a 0.8% w/v agarose gel at 15V for 16 hrs in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The DNA within the gel was then depurinated by soaking the gel twice in 0.25 M HCl for 15 min., denatured and cleaved by soaking the gel twice in 0.5 M NaOH/1.0 M NaCl 15 min., and neutralized by soaking the gel twice in 0.5M Tris pH 7.4/3M NaCl 30 min. DNA was then blotted onto a Nytran membrane (Shleicher & Shuell) by capillary transfer overnight in 6×SSC (20×SSC, 3M NaCl, 0.3M sodium citrate pH 7.0). The membrane was baked at 80° C. for 2 hrs under vacuum. Prehybridization treatment of the membrane was done in 6×SSC, 10× Denhardt's solution, 1% SDS, 50 ug/ml denatured salmon sperm DNA using 0.25 ml prehybridization solution per cm^2 of membrane. Prehybridization was carried out at 42° C. overnight.

A 32P labelled probe was prepared by random primer labelling with an Oligo Labelling Kit (Pharmacia) as per the suppliers instructions with 32P-dCTP (ICN Radiochemicals). The template DNA used was the 1055 bp BamHI fragment of pHYGI1, which is the HPT coding sequence. The fragment was gel purified and cut again with PstI (NEB) before labelling.

The hybridization was performed in 50% formamide, 6×SSC, 1% SDS, 50 ug/ml denatured salmon sperm DNA (Sigma), 0.05% sodium pyrophosphate and all of the isopropanol precipitated heat denatured probe (107 CPM/50 ug template). The hybridization was carried out at 42° C. overnight.

The membrane was washed twice in 50 ml 6×SSC, 0.1% SDS 5 min. at room temperature with shaking, then twice in 500 ml 6×. SSC, 0.1% SDS 15 min. at room temperature, then twice in 500 ml 1×SSC, 1% SDS 30 min. at 42° C., and finally in 500 ml 0.1×SSC 1% SDS 60 min. at 65° C. Membranes were exposed to Kodak X-OMAT AR film in an X-OMATIC cassette with intensifying screens. As shown in FIG. 3B, a band was observed for PH1 callus at the expected position of 1.05 Kb, indicating that the HPT coding sequence was present. No band was observed for control callus.

VI. Plant Regeneration and Production of Seed

PH1 callus was transferred directly from all of the concentrations of hygromycin used in the inhibition study to RM5 medium which consists of MS basal salts (Murashige et al. 1962) supplemented with thiamine HCl 0.5 mg/l, 2,4-D 0.75 mg/l, sucrose 50 g/l, asparagine 150 mg/l, and Gelrite 2.5 g/l (Kelco Inc. San Diego).

After 14 d on RM5 medium the majority of PH1 and negative control callus was transferred to R5 medium which is the same as RM5 medium, except that 2,4-D is omitted. These were cultured in the dark for 7 d at 26° C. and transferred to a light regime of 14 hours light and 10 hours dark for 14 d at 26° C. At this point, plantlets that had formed were transferred to one quart canning jars (Ball) containing 100 ml of R5 medium. Plants were transferred from jars to vermiculite after 14 and 21 d. Plants were grown in vermiculite for 7 or 8 d before transplanting into soil and grown to maturity. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the Ro tissue, a Southern blot was performed as previously described on leaf DNA from three randomly chosen Ro plants of PH1. As shown in FIG. 4B, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred lines A188, B73 and Oh43. Seed was harvested 45 days post-pollination and allowed to dry further 1–2 weeks. Seed set varied from 0 to 40 seeds per ear when PH1 was the female parent and from 0 to 32 seeds per ear when PH1 was the male parent.

VII. Analysis of the R1 Progeny

The presence of the hygromycin resistance trait was evaluated by a root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears.

(A) Root Elongation Bioassay

Seed was sterilized in a 1:1 dilution of commercial bleach in water plus alconox 0.1% for 20 min. in 125 ml Erlenmeyer flasks and rinsed 3 times in sterile water and imbibed overnight in sterile water containing 50 mg/ml captan by shaking at 150 rpm.

After imbibition, the solution was decanted from the flasks and the seed transferred to flow boxes (Flow Laboratories) containing 3 sheets of H_2O saturated germination paper. A fourth sheet of water saturated germination paper was placed on top of the seed. Seed was allowed to germinate 4 d.

After the seed had germinated, approximately 1 cm of the primary root tip was excised from each seedling and plated on MS salts, 20 g/l sucrose, 50 mg/l hygromycin, 0.25% Gelrite, and incubated in the dark at 26° C. for 4 d.

Roots were evaluated for the presence or absence of abundant root hairs and root branches. Roots were classified as transgenic (hygromycin resistant) if they had root hairs and root branches, and untransformed (hygromycin sensitive) if they had limited numbers of branches. The results are shown in Table 1.

(B) Etiolated Leaf Bioassay

After the root tips were excised as described above, the seedlings of one PH1 ear and one control ear were transferred to moist vermiculite and grown in the dark for 5 d. At this point 1 mm sections were cut from the tip of the coleoptile, surface sterilized 10 seconds, and plated on MS basal salts, 20 g/l sucrose, 2.5 g/l Gelrite with either 0 (control) or 100 mg/l hygromycin and incubated in the dark at 26° C. for 18 hr. Each plate contained duplicate sections of each shoot. They were then incubated in a light regimen of 14 hours light 10 hours dark at 26° C. for 48 hr, and rated on a scale of from 0 (all brown) to 6 (all green) for the percent of green color in the leaf tissue. Shoots were classified as untransformed (hygromycin sensitive) if they had a rating of zero and classified as transformed (hygromycin resistant) if they had a rating of 3 or greater. The results are shown in Table 3.

(C) Southern Blots

Seedlings from the bioassays were transplanted to soil and are growing to sexual maturity. DNA was isolated from 0.8 g of leaf tissue after about 3 weeks and probed with the HPT coding sequence as described previously. Plants with a 1.05 Kb band present in the Southern blot were classified as transgenic. As shown in FIG. 5B and FIG. 5C, two out of seven progeny of PH1 plant 3 were transgenic as were three out of eight progeny of PH1 plant 10. The blot results correlated precisely with data from the bioassays, confirming that the heterologous DNA was transmitted through one complete sexual life cycle. All data are summarized in Table 3.

TABLE 3

ANALYSIS OF PH1 R1 PLANTS

PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.1	+	ND	+	4.1	–	ND	ND
3.2	–	ND	–	4.2	–	ND	ND
3.3	–	ND	–	4.3	–	ND	ND
3.4	–	ND	–	4.4	–	ND	ND
3.5	–	ND	–	4.5	–	ND	ND
3.6	+	ND	+	4.6	–	ND	ND
3.7	–	ND	–	4.7	–	ND	ND
				2.1	–	ND	–
10.1	+	+	+	1.1	–	–	–
10.2	+	+	+	1.2	–	–	ND
10.3	–	–	ND	1.3	–	–	ND
10.4	–	–	–	1.4	–	–	ND
10.5	–	–	–	1.5	–	–	ND
10.6	–	–	–	1.6	–	–	ND
10.7	–	–	–	1.7	–	–	ND
10.8	ND	+	+	1.8	–	–	ND

KEY:
+ = transgenic;
– = nontransgenic;
ND = not done

EXAMPLE II

The procedure of Example I was repeated with minor modifications.

I. Plant Lines and Tissue Cultures

The embryogenic maize callus line, AB12, was used as in Example I. The line had been initiated about 18 months before the actual bombardment occurred.

II. Plasmids

The plasmids pBII221 and pHYGI1 described in Example I were used.

III. DNA Delivery Process

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5 M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. One tube contained only plasmid pBII221; two tubes contained only plasmid pHYGI1; and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

2 × pBII221 prep	For transient expression
7 × pHYGI1 prep	Potential positive treatment
3 × TE prep	Negative control treatment

After all the bombardments were performed, the callus from the pBII221 treatments was transferred plate for plate to F medium as five 50 mg pieces. After 2 d the callus was placed into GUS assay buffer as per Example I. Numbers of transiently expressing cells were counted and found to be 686 and 845 GUS positive cells, suggesting that the particle delivery process had occurred in the other bombarded plates.

IV. Selection of Transformed Callus

After bombardment the callus from the pHYGI1 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate (different from Example I). The same was done for two of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 13 d the callus on round 1 selection plates was indistinguishable from unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin. An approximate five fold expansion of the numbers of plates occurred.

The callus on round 2 selection plates had increased substantially in weight after 23 d, but at this time appeared close to dead. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin. This transfer of all material from round 2 to round 3 selection differs from Example I in which only viable sectors were transferred and the round 2 plates reserved.

At 58 d post-bombardment three live sectors were observed proliferating from the surrounding dead tissue. All three lines were from pHYGI1 treatments and were designated 24C, 56A, and 55A.

After 15 d on maintenance medium, growth of the lines was observed. The line 24C grew well whereas lines 55A and 56A grew more slowly. All three lines were transferred to F medium containing 60 mg/l hygromycin. Unselected control callus from maintenance was plated to F medium having 60 mg/l hygromycin.

After 19 d on 60 mg/l hygromycin the growth of line 24C appeared to be entirely uninhibited, with the control showing approximately 80% of the weight gain of 24C. The line 56A was completely dead, and the line 55A was very close. The lines 24C and 55A were transferred again to F 60 mg/l hygromycin as was the control tissue.

After 23 d on 60 mg/l hygromycin the line 24C again appeared entirely uninhibited. The line 55A was completely dead, as was the negative control callus on its second exposure to F 60 mg/l hygromycin.

At 88 d post-bombardment, a sector was observed proliferating among the surrounding dead tissue on the round 3 selection plates. The callus was from a plate bombarded with pHYGI1 and was designated 13E. The callus was transferred to F medium and cultured for 19 d. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin. After 14 d of culture, the callus line 13E appeared uninhibited on both levels of hygromycin. The control callus appeared to have about 80% of the weight gain of 13E. The callus lines were transferred to fresh media at the same respective levels of hygromycin.

V. Confirmation of Transformed Callus

A Southern blot was prepared from DNA from the line 24C. As shown in FIG. 6B, a band was observed for the line 24C at the expected size of 1.05 Kb showing that the line 24C contained the HPT coding sequence. No band was observed for DNA from control tissue. The name of the callus line 24C was changed to PH2.

VI. Plant Regeneration and Production of Seed

The line 24C along with unselected control callus were placed onto RM5 medium to regenerate plants as in Example I. After 16 d the callus was transferred to R5 medium as in Example I.

EXAMPLE III

The procedure of Example II was repeated exactly except that different plasmids were used.

The plasmids pBII221 and pHYGI1 described in Example I were used as well as pMS533 which is a plasmid that contains the insecticidal *Bacillus thuringiensis* endotoxin (BT) gene fused in frame with the neomycin phosphotransferase (NPTII) gene. 5' of the fusion gene are located segments of DNA from the CaMV 35S and nopaline synthase promoters. 3' from the fusion gene are segments of DNA derived from the tomato protease inhibitor I gene and the poly A region of the nopaline synthase gene.

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. Two tubes contained plasmids pHYGI1 and pMS533 and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

9 × pHYGI1/pMS533	Potential positive treatment
2 × TE prep	Control treatment

After bombardment the callus from the pHYGI1/pMS533 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate. The same was done for one of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 12 d the callus on round 1 selection plates appeared to show about 90% of the weight gain of the unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin as ten 30 mg pieces per plate.

After 22 d of selection on round 2 selection plates, the callus appeared completely uninhibited. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin.

At 74 d post-bombardment a single viable sector was observed proliferating from the surrounding necrotic tissue. The callus line was from pHYGI1/pMS533 treated material and was designated 86R. The callus line 86R was transferred to F medium.

After 24 d the callus line 86R had grown substantially. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin.

After 19 d of culture, the callus line 86R appeared to grow rapidly and was uninhibited on both levels of hygromycin. The control callus appeared to have only about 50% of the weight gain of 86R. The callus lines were transferred to fresh media at the same respective levels of hygromycin to further test the resistance of the callus line 86R to hygromycin.

COMPARATIVE EXAMPLE A

The basic procedures of Examples I–III have been attempted except varying the selection regime or the form of the callus. These other attempts, which are detailed in Table 4 below, were not successful. Since they were not repeated several times, it is not known whether they can be made to work. In all of the procedures, no viable sectors were observed. In the Table, "Sieved" indicates that the callus was

passed through an 860 micron sieve before bombardment; the selective agent was hygromycin for each case except when pMXTI1 was the plasmid and methotrexate the selection agent.

TABLE 4

Summary of Comparative Example A

Recip. Tissue	Plasmids	Recov. Period	Round 1 Level	Round 1 Period	Round 2 Level	Round 2 Period
Clumps	pCHN1-1 pBII221	13	60	21	60	81
Clumps	pCHN1-1 pBII221	14	100	22	—	—
Clumps	pHYGI1 pBII221	8	60	19	30	132
Clumps	pCHN1-1 pBII221	0	30	22	60	109
Clumps	pMTXI1 pBII221	8	3	103	—	—
Sieved	pCHN1-1 pBII221	13	—	—	—	—

What is claimed is:

1. A method for producing a fertile transgenic *Zea mays* plant, comprising the steps of:

(i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, wherein said DNA comprises a selectable marker gene;

(ii) selecting a population of transformed *Zea mays* cells; and

(iii) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is heritable, to yield transgenic progeny *Zea mays* plants.

2. The method of claim 1 wherein the fertile transgenic *Zea mays* plant is regenerated from transformed embryogenic tissue.

3. The method of claim 1 wherein the cells are derived from immature embryos.

4. A method of producing a fertile transgenic *Zea mays* plant, comprising:

(a) cultivating a fertile transgenic *Zea mays* plant produced by a process comprising

(i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, said DNA comprising a selectable marker gene;

(ii) selecting a population of transformed *Zea mays* cells; and

(iii) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is heritable, to yield a transgenic progeny *Zea mays* plant; and

(b) obtaining said transgenic progeny *Zea mays* plant, the genome of which has been altered by said DNA, wherein said DNA is expressed so that the transgenic progeny plant exhibits one or more phenotypic characteristics that render it identifiable over the corresponding untransformed *Zea mays* plant which does not comprise said DNA construct, and wherein the DNA is transmitted through a complete sexual cycle of the transgenic progeny *Zea mays* plant to progeny plants.

5. A method of producing a fertile transgenic inbred *Zea mays* plant, comprising:

(a) cultivating a fertile transgenic *Zea mays* plant produced by a process comprising

(i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, said DNA comprising a selectable marker gene;

(ii) selecting a population of transformed *Zea mays* cells; and

(iii) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is heritable, to yield transgenic progeny *Zea mays* plants;

(b) crossing a member of a nontransgenic inbred *Zea mays* line to said transgenic progeny plant;

(c) recovering a fertile transgenic *Zea mays* progeny plant that comprises said DNA; and

(d) repeating steps (b) and (c) to yield a fertile transgenic inbred *Zea mays* progeny, the genome of which has been altered by said DNA, wherein said DNA is expressed so that the transgenic inbred *Zea mays* plant exhibits one or more phenotypic characteristics that render it identifiable over the corresponding untransformed *Zea mays* plant which does not comprise said DNA, and wherein the DNA is transmitted through a complete sexual cycle of the transgenic inbred *Zea mays* plant to progeny plants.

6. The method of claim 5 wherein said phenotypic characteristic is herbicide resistance.

7. The method of claim 5 wherein said phenotypic characteristic is insect resistance.

8. The method of claim 7 wherein said DNA construct encodes a *Bacillus thuringiensis* endotoxin.

9. A method of producing a fertile transgenic hybrid *Zea mays* plant, comprising:

(a) cultivating a fertile transgenic *Zea mays* plant produced by a process comprising

(i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, wherein the DNA comprises a selectable marker gene;

(ii) selecting a population of transformed *Zea mays* cells; and

(iii) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is heritable, to yield transgenic *Zea mays* progeny plants;

(b) crossing a non-transgenic inbred *Zea mays* line with said transgenic *Zea mays* plant; and

(c) recovering said fertile transgenic hybrid *Zea mays* plant, the genome of which has been altered by the introduction of said DNA, wherein said DNA is expressed so that the transgenic hybrid *Zea mays* plant exhibits one or more phenotypic characteristics that render it identifiable over the corresponding untransformed *Zea mays* plant which does not comprise said DNA, and wherein the DNA is transmitted through a complete sexual cycle of the transgenic hybrid *Zea mays* plant to progeny plants.

10. The method of claim 9 wherein said phenotypic characteristic is herbicide resistance.

11. The method of claim 9 wherein said phenotypic characteristic is insect resistance.

12. The method of claim 11 wherein said DNA construct encodes a *Bacillus thuringiensis* endotoxin.

13. The process of any one of claim 1, 4, 5 or 9 wherein the selectable marker gene encodes antibiotic resistance.

14. The process of claim 13 wherein the gene encodes resistance to bleomycin.

15. The process of claim 13 wherein the gene encodes resistance to hygromycin.

16. The process of claim 15 wherein the gene is the hpt gene.

17. The process of claim 13 wherein the gene encodes resistance to neomycin, kanamycin or G418.

18. The process of claim 17 wherein the gene is the nptII gene.

19. The process of claim 17 wherein the gene is the nptI gene.

* * * * *

EXHIBIT B

US005538877A

United States Patent [19]

Lundquist et al.

[11] Patent Number: 5,538,877

[45] Date of Patent: Jul. 23, 1996

[54] **METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS**

[75] Inventors: **Ronald C. Lundquist**, Minnetonka; **David A. Walters**, Bloomington, both of Minn.

[73] Assignee: **DeKalb Genetics Corporation**, St. Paul, Minn.

[21] Appl. No.: **974,379**

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Related U.S. Application Data

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[52] **U.S. Cl.** **435/172.3**; 435/172.1; 435/240.48; 435/240.49; 935/52; 935/55; 935/67; 935/85; 800/205; 800/235; 800/DIG. 56

[58] **Field of Search** 435/172.1, 172.3, 435/240.4, 240.45, 240.49, 52, 54, 55, 67, 85; 800/205

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,370,160	1/1983	Ziemelis	504/323
4,559,302	12/1985	Ingolia	435/172.3
4,581,847	4/1986	Hibberd et al.	47/58
4,666,844	5/1987	Cheng	435/240.5
4,727,028	2/1988	Santerre et al.	435/240.2
4,806,483	2/1989	Wang	435/240.49
4,830,966	5/1987	Close	435/240.49
4,940,835	7/1990	Shah et al.	800/205
5,049,500	9/1991	Arnizen et al.	435/172.3

FOREIGN PATENT DOCUMENTS

126537	4/1983	European Pat. Off. .	
141373	5/1985	European Pat. Off. .	
154204	9/1985	European Pat. Off. .	
160390	11/1985	European Pat. Off. .	
0193259	9/1986	European Pat. Off.	C12M 15/00
202668	11/1986	European Pat. Off. .	
204549	12/1986	European Pat. Off. .	
242236	10/1987	European Pat. Off. .	
242246	10/1987	European Pat. Off. .	
299552	1/1988	European Pat. Off. .	
262971	4/1988	European Pat. Off. .	
275069	7/1988	European Pat. Off. .	
270356	8/1988	European Pat. Off. .	
280400	8/1988	European Pat. Off. .	
282164	9/1988	European Pat. Off. .	
292435	11/1988	European Pat. Off. .	
290395	11/1988	European Pat. Off. .	
289479	11/1988	European Pat. Off. .	
301749	2/1989	European Pat. Off. .	
334539	9/1989	European Pat. Off. .	
331855	9/1989	European Pat. Off. .	
348348	12/1989	European Pat. Off. .	
442174	8/1991	European Pat. Off. .	
3738874	11/1988	Germany .	
8801444	1/1990	Netherlands .	
2159173	11/1985	United Kingdom .	
WO85/01856	5/1985	WIPO .	
WO85/02972	7/1985	WIPO .	
WO87/05629	9/1987	WIPO .	
89/04371	5/1988	WIPO .	
WO89/12102	12/1989	WIPO .	
WO90/10691	9/1990	WIPO .	

OTHER PUBLICATIONS

T. Nelson, *The Plant Cell*, 2, 589 (Jul. 1990).

C. Rhodes, *Bio/Technology*, 7, 548 (Jun. 1989).

I. Potrykus, *Bio/Technology*, 8, 540 (Jun. 1990).

Agricell Report, news article entitled "'Bullets' Transform Plant Cells", p. 5 (Jul. 1987).

H. Ahokes, "Electrophoretic Transfection of Cereal Grains with Exogenous Nucleic Acid", *Soc. Biochem. Biophys., Microbio, Fen., Biotieteen Paivat (Bioscience Days)*, Abstracts, Technical University of Helsinki, Espoo, p. 2 (1989).

C. Armstrong et al., "Genetic and Cytogenetic Variation in Plants Regenerated from Organogenic and Friable, Embryogenic Tissue Cultures of Maize", *Biol. Abstracts*, vol. 85, Abstract 117662 (1988).

R. Barker et al., "Nucleotide Sequence of the T-DNA Region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi5955", *Plant Mol. Biol.*, 2:335–350 (1983).

M. Bevan et al., "A Chimearic Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation", *Nature*, 304:184–187 (1983).

Cao et al., "Transformation of Rice and Maize Using the Biolistic Process", *Plant Gene Transfer*, 129:21–33 (1990).

V. Chandler et al., "Two Regulatory Genes of the Maize Anthocyanin Pathway Are Homologous: Isolation of B Utilizing R Genomic Sequences", *The Plant Cell*, 1:1175–1183 (1989).

Creissen et al., "*Agrobacterium*—and microprojectile –Mediated Viral DNA Delivery into Barley Microspore–Derived Cultures", *Plant Cell Reports*, 8:680–683 (Apr. 1990).

Christou et al., "Cotransformation Frequencies of Foreign Genes in Soybean Cell Cultures", *Theor. Appl. Genet.*, 79:337–341 (1990).

A. Crossway et al., "Integration of Foreign DNA following Microinjection of Tobacco Mesophyll Protoplasts", *Mol. Gen. Genet.*, 202:179–185 (1986).

M. De Block et al., "Engineering Herbicide Resistance in Plants by Expression of a Detoxifying Enzyme", *EMBO J.*, 6:2513–2518 (1987).

(List continued on next page.)

Primary Examiner—Gary Benzion

Attorney, Agent, or Firm—Schwegman, Lundberg, Woessner & Kluth

[57] **ABSTRACT**

Fertile transgenic *Zea mays* (corn) plants which stably express heterologous DNA which is heritable are disclosed along with a process for producing said plants. The process comprises the microjectile bombardment of friable embryogenic callus from the plant to be transformed. The process may be applicable to other graminaceous cereal plants which have not proven stably transformable by other techniques.

10 Claims, 10 Drawing Sheets

OTHER PUBLICATIONS

W. De Greef et al., "Evaluation of Herbicide Resistance in Transgenic Crops Under Field Conditions", *Bio/Technology,* 7:61–64 (1989).

R. Dekeyser et al., "Evaluation of Selectable Markers for Rice Transformation", *Plant Physiol.,* 90:217–223 (1989).

DeWald et al. "Plant Regeneration from Inbred Maize Suspensions", Abstract Al–36, *7th International Congress on Plant Tissue and Cell Culture,* Amsterdam, Jun. 24–29 (1990).

P. Evans, "Somaclonal Variation—Genetic Basis and Breeding Applications", *Trends Genet.,* 5:46–50 (1989).

P. Fransz, *Cytodifferentiation During Callus Initiation and Somatic Embryogenesis in Zea Mays L.,* Ph.D. Thesis, U. of Wageningen Press, The Netherlands (1988).

M. Fromm et al., "Expression of Genes Transferred into Monocot and Dicot Plant Cells by Electroporation", *PNAS,* 82:5824–5828 (1985).

Gen. Eng. News, news article entitled "Shotgunning DNA into Cells" (Jul./Aug. 1987).

O. Gould et al., "Shoot Tip Culture as a Potential Transformation Systems", *Beltwide Cotton Production Research Conferences,* New Orleans, LA, Abstract p. 91 (Jan. 3–8, 1988).

A. Graves et al., "The Transformation of *Zea mays* Seedlings with *Agrobacterium tumefaciens", Plant Mol. Biol.,* 7:43–50 (1986).

C. Green et al., "Plant Regeneration from Tissue Cultures of Maize", *Crop Science,* 15:417–421 (1975).

C. Green et al., "Somatic Cell Genetic Systems in Corn", *Advances in Gene Technology: Molecular Genetics of Plants and Animals,* Academic Press, Inc., 147–157 (1983).

P. Hooykaas, "Transformation of plant cells via *Agrobacterium", Plant Mol. Biol.,* 13:327–336 (1989).

M. Horn et al., "Transgenic Plants of Orchard Grass (*Dactylis glomerata* L.) from Protoplasts", *Chem Abstracts,* 110:208, Abstract 89869A (1989).

R. Jefferson et al., "–Glucuronidase from *Escherichia coli* as a Gene–Fusion Marker", *PNAS,* 83:8447–8451 (1986).

R. Jefferson, "Assaying Chimeric Genes in Plants: The GUS Gene Fusion System", *Plant Mol. Biol. Reporter,* 5:387–405 (1987).

K. Kamo et al., "Establishment and Characterization of Long–Term Embryogenic Maize Callus and Cell Suspension Cultures", *Plant Science,* 45:111–117 (1986).

K. Kartha et al., "Transient Expression of Chloramphenicol Acetyltransferase (CAT) Gene in Barley Cell Cultures and Immature Embryos Through Microprojectile Bombardment", *Plant Cell Reports,* 8:429–432 (1989).

T. Klein et al., "Regulation of anthocyanin biosynthetic genes introduced into intact maize tissues by microprojectiles", *PNAS,* 86:6682–6685 (1989).

T. Klein et al., "Genetic Transformation of Maize Cells by Particle Bombardment and the Influence of Methylation on Foreign–Gene Expression", *Gene Manipulation in Plant Improvement II,* Plenum Press, NY, pp. 265–266 (1990).

M. Kozak, "Point Mutations Define a Sequence Flanking the AUG Initiator Codon that Modulates Translation by Eukaryotic Ribosomes", *Cell,* 44:283–292 (1986).

P. Lazzeri et al., "In Vitro Genetic Manipulation of Cereals and Grasses", *Ad. Cell Culture,* 6:291–293 (1988).

H. Lorz et al., "Advances in Tissue Culture and Progress Towards Genetic Transformation of Cereals", *Plant Breeding,* 100:1–25 (1988).

S. Ludwig et al., "High Frequency Callus Formation from Maize Protoplasts", *Theor. Appl. Genet.,* 71:344–350 (1985).

S. Ludwig et al., "Lc, A Member of the Maize R Gene Family Responsible for Tissue–Specific Anthocyanin Production, Encodes a Protein Similar to Transcriptional Activators and Contains the myc–Homology Region", *PNAS,* 86:7092–7096 (1989).

S. Ludwig et al., "A Regulatory Gene as a Novel Visible Marker for Maize Transformation", *Science,* 247:449–450 (Jan. 26, 1990).

S. Ludwig et al., "Maize R Gene Family: Tissue–Specific Helix–Loop–Helix Proteins", *Cell,* 62:849–851 (Sep. 7, 1990).

H. Lutcke et al., "Selection of AUG Initiation Condons Differs in Plants and Animals", *EMBO J.,* 6:43–48 (1987).

C. McDaniel et al., "Cell–Lineage Patterns in the Shoot Apical Meristem of the Germinating Maize Embryo", *Planta,* 175:13–22 (1988).

M. Meadows, "Characterization of Cells and Protoplasts of the B73 Maize Cell Line", *Plant Sci. Letters,* 28:337–348 (1982/83).

R. Mendel et al., "Delivery of Foreign Genes to Intact Barley Cells by High–Velocity Microprojectiles", *Theor. Appl. Genet.,* 78:31–34 (1989).

T. Murakami et al., "The Bialaphos Biosynthetic Genes of *Streptomyces hygroscopicus*: Molecular Cloning and Characterization of the Gene Cluster", *Mol. Gen. Genet.,* 205:42–50 (1986).

J. Odell et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter", *Nature,* 313:810–811 (1985).

P. Ozias–Akins et al., "Progress and Limitations in the Culture of Cereal Protoplasts", *Trends in Biotechnology,* 2:119–123 (1984).

P. Ozias–Akins et al., "In Vitro Regeneration and Genetic Manipulation of Grasses", *Physiologia Plantarum,* 73:565–569 (1988).

J. Poehlman, *Breeding Field Crops,* 3rd Ed., AVI Publishing Co., pp. 469–481 (1986).

I. Potrykus et al., "Callus Formation from Stem Protoplasts of Corn (*Zea mays* L.)", *Molec. gen. Genet.,* 156:347–350 (1977).

Sanford, "Biolistic Plant Transformation", *Physiol. Plantarum,* 79:206–209 (1990).

A. Schmidt et al., "Media and Environmental Effects on Phenolics Production from Tobacco Cell Cultures", *Chem Abstracts,* 110:514–515, Abstract 230156 (1989).

R. Smith et al., "Shoot Apex Explant for Transformation", *Plant Physiology (Suppl.),* 86:108, Abstract 646 (1988).

Spencer et al. *Poster Presentation, Faseb Plant Gene Expression Conference,* Copper Mountain, Colorado, (Aug. 6–11 1989).

Spencer et al., "Bialaphos Selection of Stable Transformations from Maize Cell Culture", *Theor. Appl. Genet.,* 79:625–631 (May 1990).

C. Thompson et al., "Characterization of the Herbicide–Resistance Gene bar from *Streptomyces hygroscopicus", EMBO J.,* 6:2519–2523 (1987).

Tomes et al., "Transgenic Tobacco Plants and Their Progeny Derived by Microprojectile Bombardment of Tobacco Leaves", *Plant Mol. Biol.,* 14:261–268 (Feb. 1990).

D. Twell et al., "Transient Exression of Chimeric Genes Delivered into Pollen by Microprojectile Bombardment", *Plant Physiol.,* 91:1271–1274 (1989).

E. Ulian et al., "Transformation of Plants via the Shoot Apex", *In Vitro Cellul & Dev. Biol.*, 9:951–954 (1988).
V. Vasil et al., "Improved Efficiency of Somatic Embryogenesis and Plant Regeneration in Tissue Cultures of Maize (*Zea mays* L.)", *Theor. Appl. Genet.*, 68:285–289 (1983).
V. Vasil et al., "Plant Regeneration from Friable Embryogenic Callus and Cell Suspension Cultures of *Zea mays* L.", *J. Plant Physiol.*, 124:399–408 (1986).
V. Walbot et al., "Molecular Genetics of Corn", *Ag. Mono.*, 18:389–430 (1988).
Y. Wang et al., "Transient Expression of Foreign Genes in Rice, Wheat and Soybean Cells Following Particle Bombardment", *Plant Mol. Biol.*, 11:433–439 (1988).
J. White et al., "A Cassette Containing the Bar Gene of *Streptomyces hygroscopicus*: A Selectable Marker for Plant Transformation", *Nucleic Acids Res.*, 18:1062 (1989).
McCabe et al. (1988) Biotechnology vol. 6 pp. 923–926.
I. Potrykus, *Trends in BioTechnology*, 7, 269 (1989).
K. Weising et al., *Ann. Rev. Genet.*, 22, 421 (1988).
E. C. Cocking et al., *Science*, 236, 1259 (1987).
A. C. F. Graves et al., *Plant Molecular Biology*, 7, 43 (1986).
C. A. Rhodes et al., *Science*, 240, 204 (1988).
J. C. Sanford et al., *Particulate Science and Technology*, 5, 27 (1987).
T. M. Klein et al., *PNAUS USA*, 85, 4305 (1988).
T. M. Klein et al., *Plant Physiol.*, 91, 400 (1989).
R. L. Phillips et al., in *Corn and Corn Improvement*, ASA et al., pubs., Madison, WI (3d ed., 1988) at Chapter 5.
P. Christou et al., *Plant Physiol.*, 87, 671 (1988).T. M. Klein et al., *Nature*, 327, 70 (1987).
C. E. Green et al., in *Maize for Biological Research*, Plant Molec. Biol. Assoc. (1982) at pp. 367–372.
J. Callis et al., *Genes and Development*, 1, 1183 (1987).
R. A. Jefferson et al., *EMBO J.*, 6, 3901 (1987).
T. M. Klein et al., *Bio/Technology*, 6, 559 (1988).
M. Neuffer et al., in *Maize for Biological Research*, Plant Molec. Biol. Assoc. (1988) at pp. 19–30.
L. Gritz et al., *Gene*, 25, 179 (1983).
M. Bevan et al., *Nuc. Acids Res.*, 11, 369 (1983).
H. Guilley et al., *Cell*, 30, 763 (1982).
J. R. de Wett et al., *PNAS USA*, 82, 7870 (1985).
J. C. Sanford et al., *Theor. Appl. Genet.*, 69, 571 (1985).
Y. Ohta, *PNAS USA*, 83, 715 (1986).
G. Booy et al., *J. Plant Physiol.*, 135, 319 (1989).
M. E. Fromm et al., *Nature*, 319 791 (1986).
C. A. Rhodes et al., *Bio/Technology*, 6, 56 (Jan. 1988).
R. D. Shillito et al., *Bio/Technology*, 7, 581 (Jun. 1989).
L. M. Pribli et al., *Bio/Technology*, 7, 589 (Jun. 1989).
N. Grimsley et al., *Mol. Gen. Genet.*, 21, 309 (1989).
I. Potrykus, *Bio/Technology*, 535 (Jun. 1990).

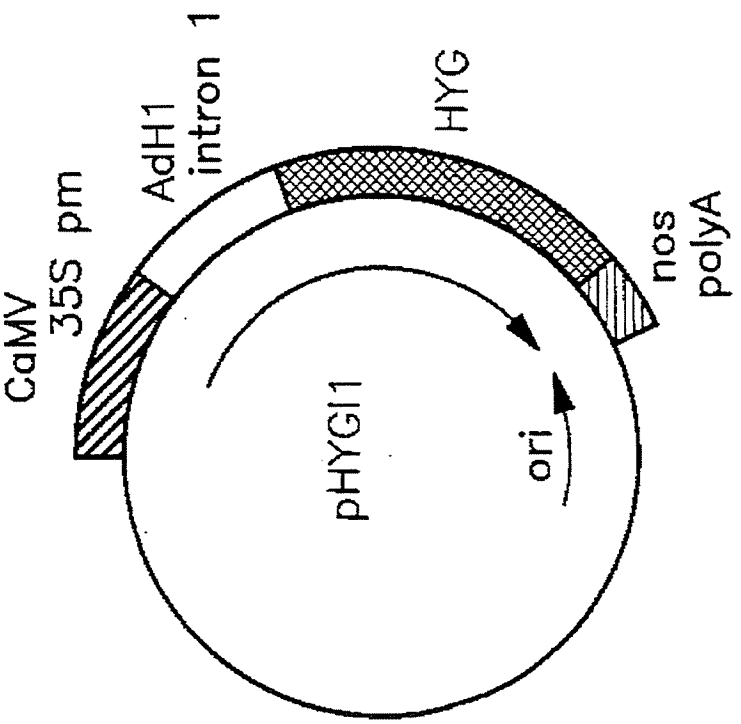

CaMV
35S pm
AdH1
intron 1
HYG
nos
polyA
pHYG11
ori

FIG. 1A

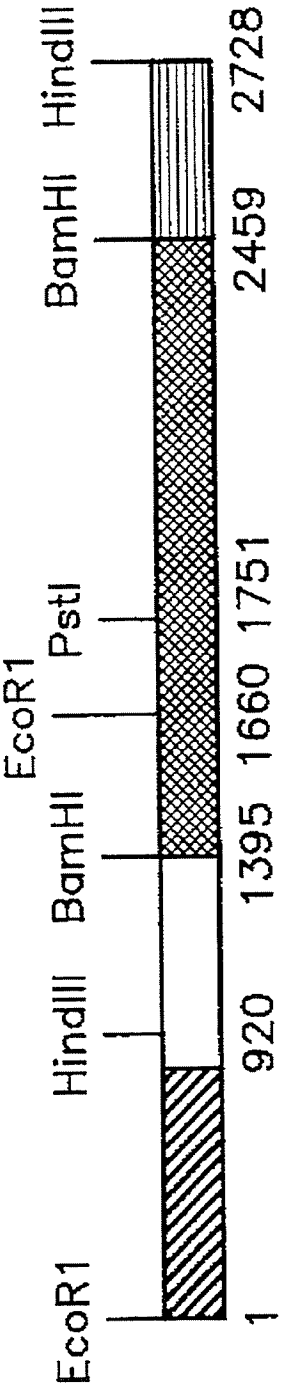

EcoR1
HindIII
BamHI
EcoR1
PstI
BamHI
HindIII
BamHI
1
920
1395
1660
1751
2459
2728

FIG. 1B

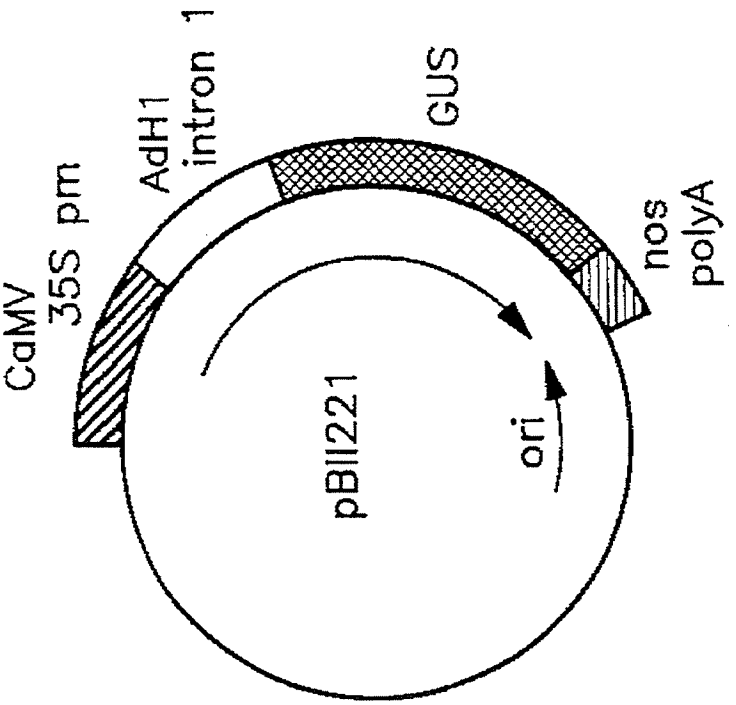
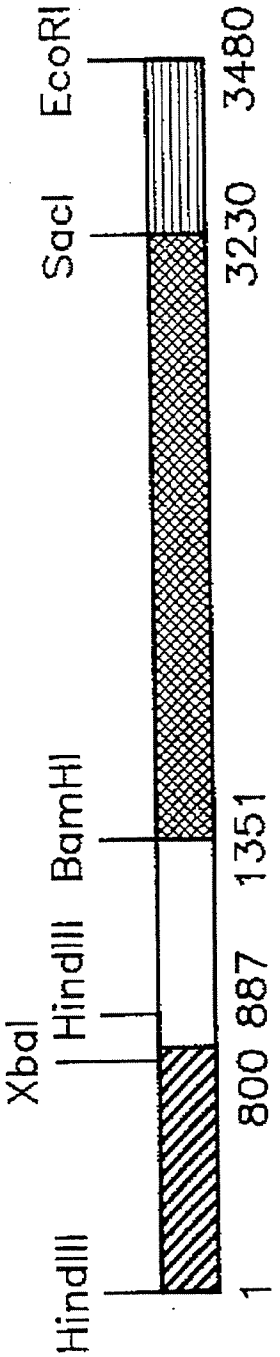

CaMV
35S pm
AdH1
intron 1
GUS
nos
polyA
pBII221
ori

FIG. 2A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SacI
3230
BamHI
1351
XbaI
HindIII
887
800
HindIII
1

FIG. 2B

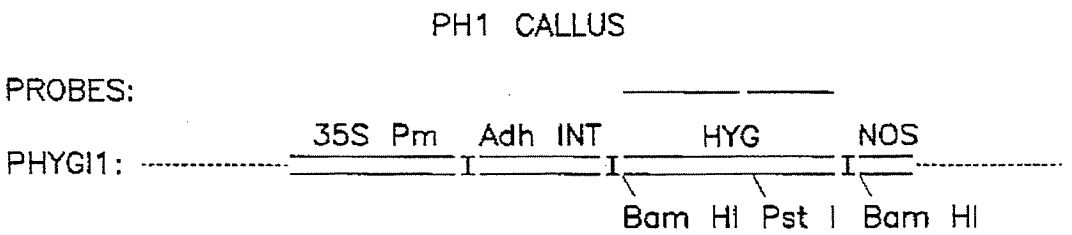

PH1 CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 3A

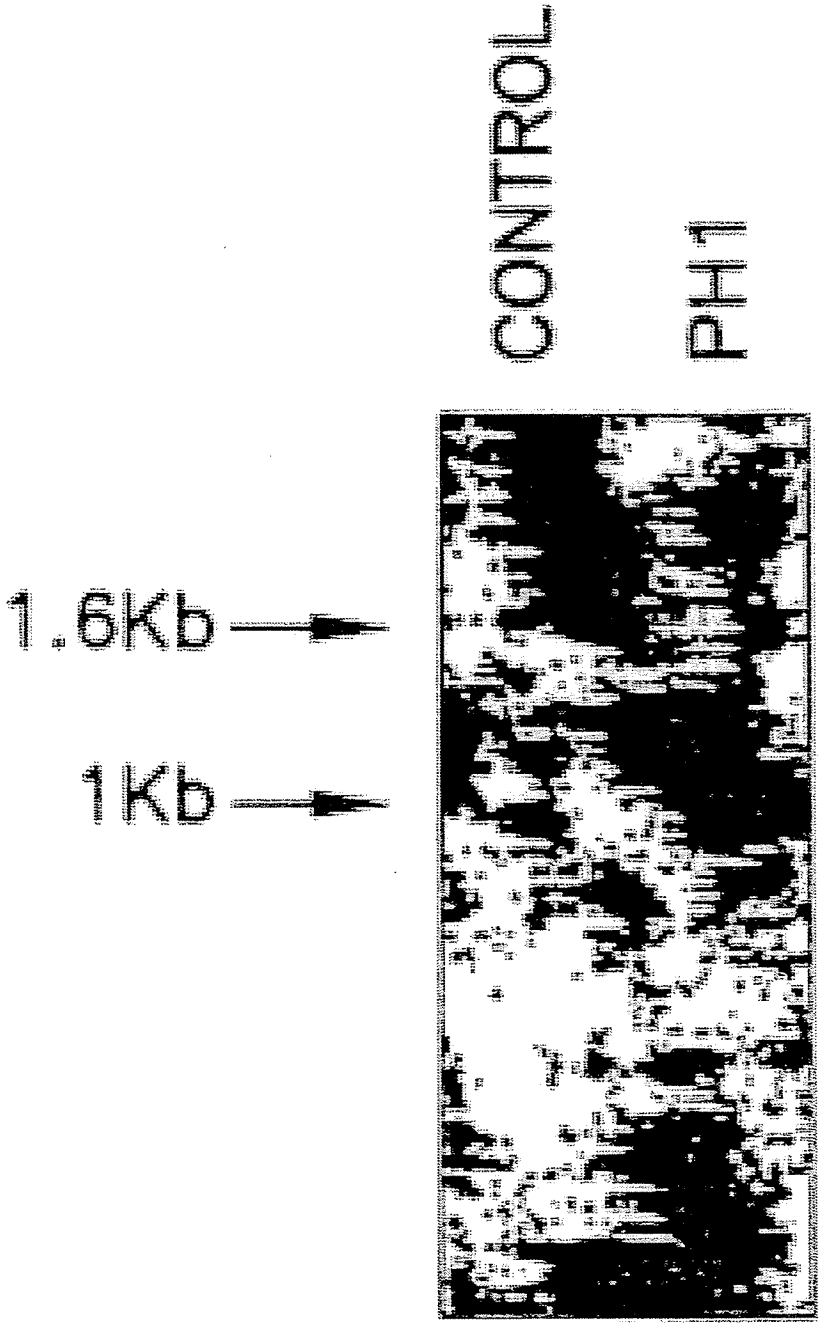


FIG. 3B

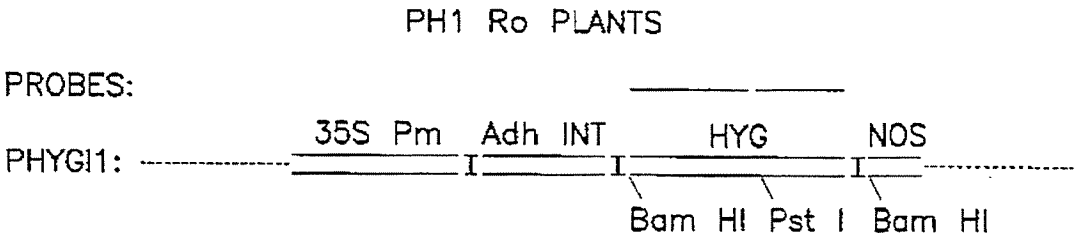

PH1 Ro PLANTS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 4A

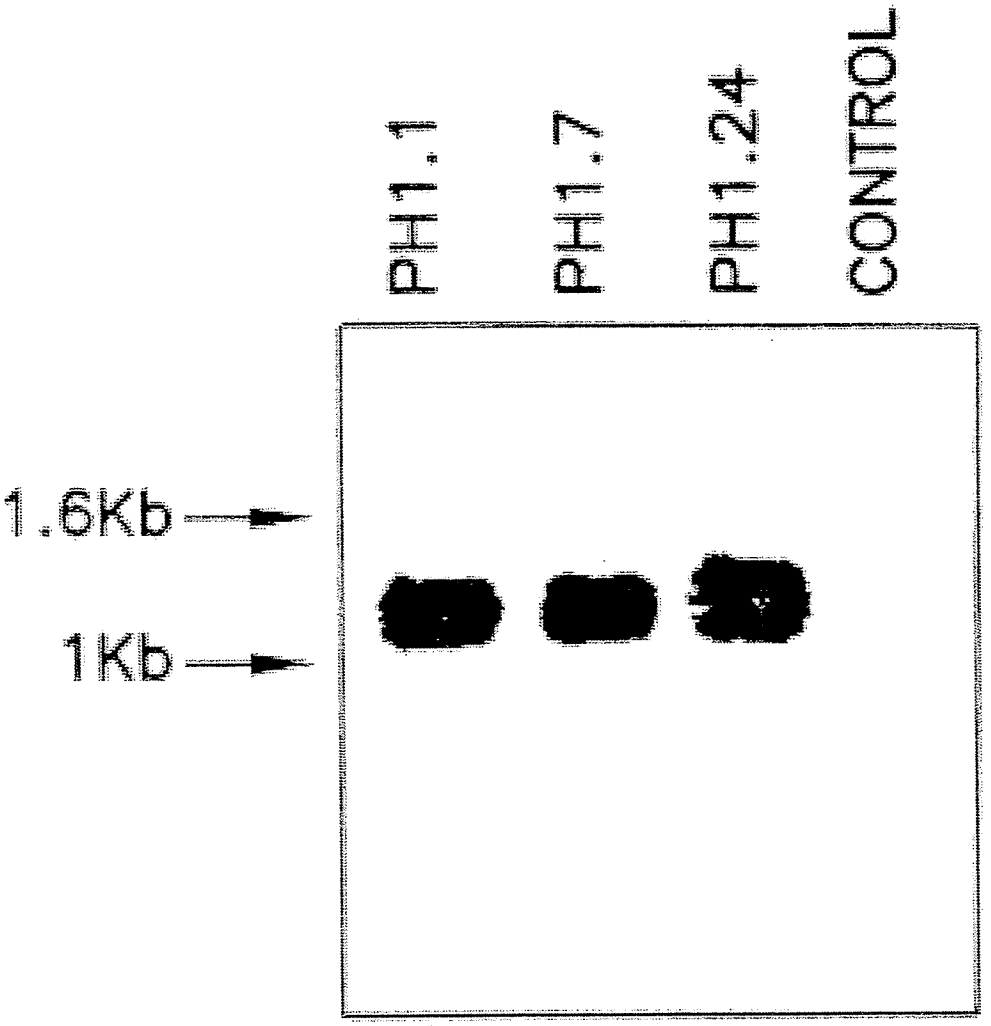


FIG. 4B

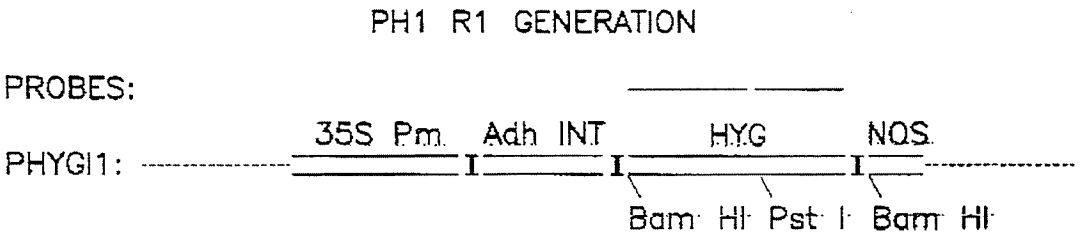

PH1 R1 GENERATION
PROBES:
PHYGI1:
35S Pm.
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 5A

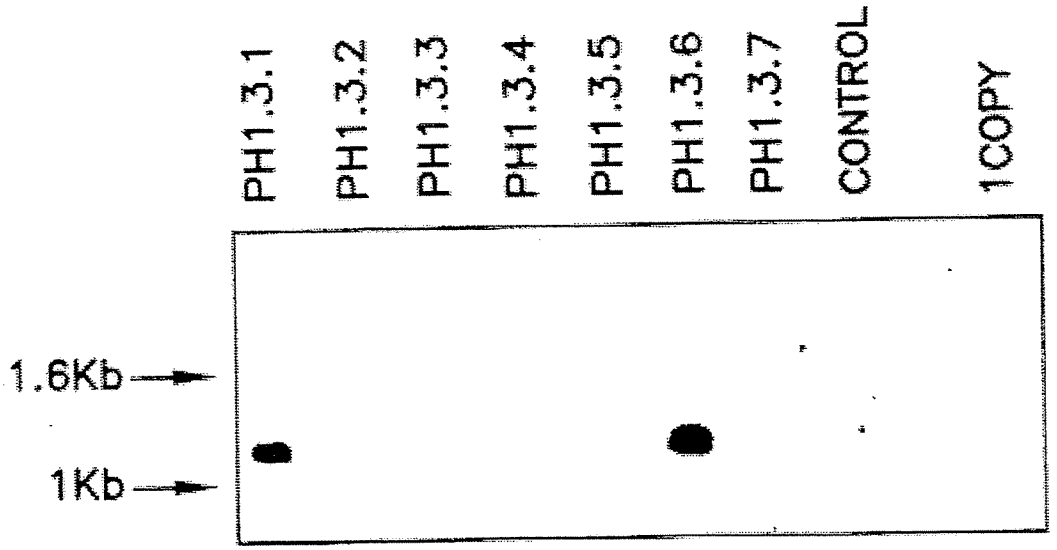

PH1.3.1
PH1.3.2
PH1.3.3
PH1.3.4
PH1.3.5
PH1.3.6
PH1.3.7
CONTROL
1COPY
1.6Kb
1Kb

FIG. 5B

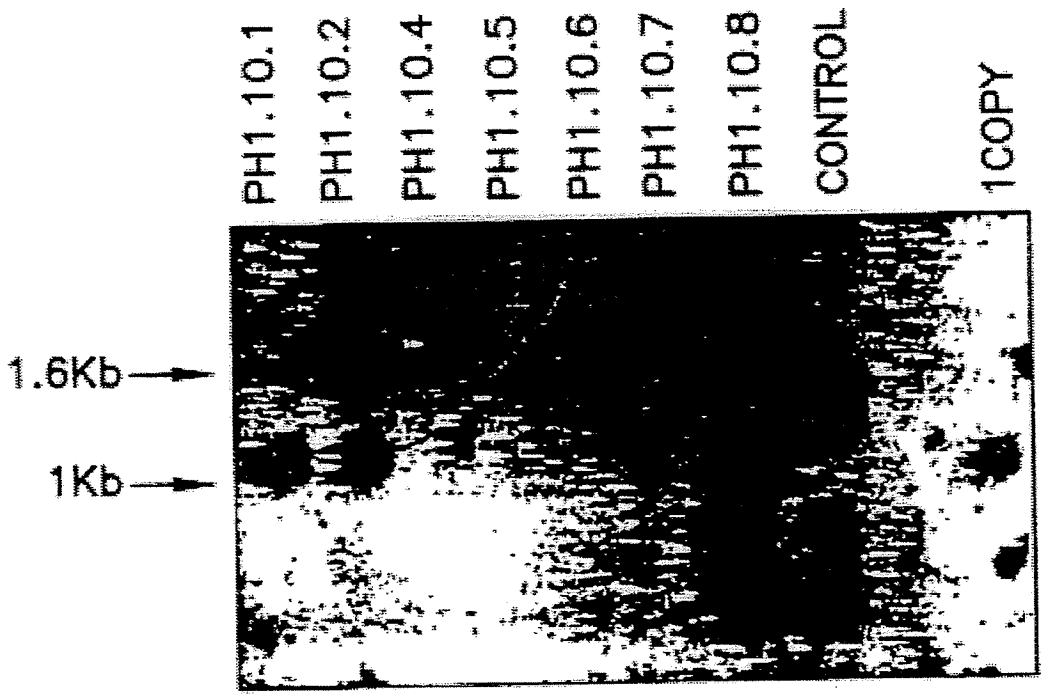

PH1.10.1
PH1.10.2
PH1.10.4
PH1.10.5
PH1.10.6
PH1.10.7
PH1.10.8
CONTROL
1COPY
1.6Kb
1Kb

FIG. 5C

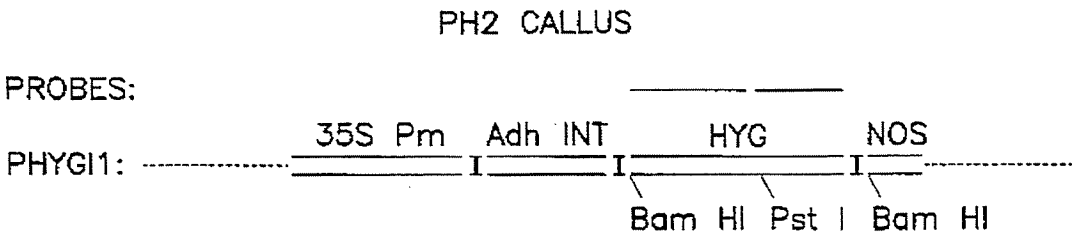

PH2 CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 6A

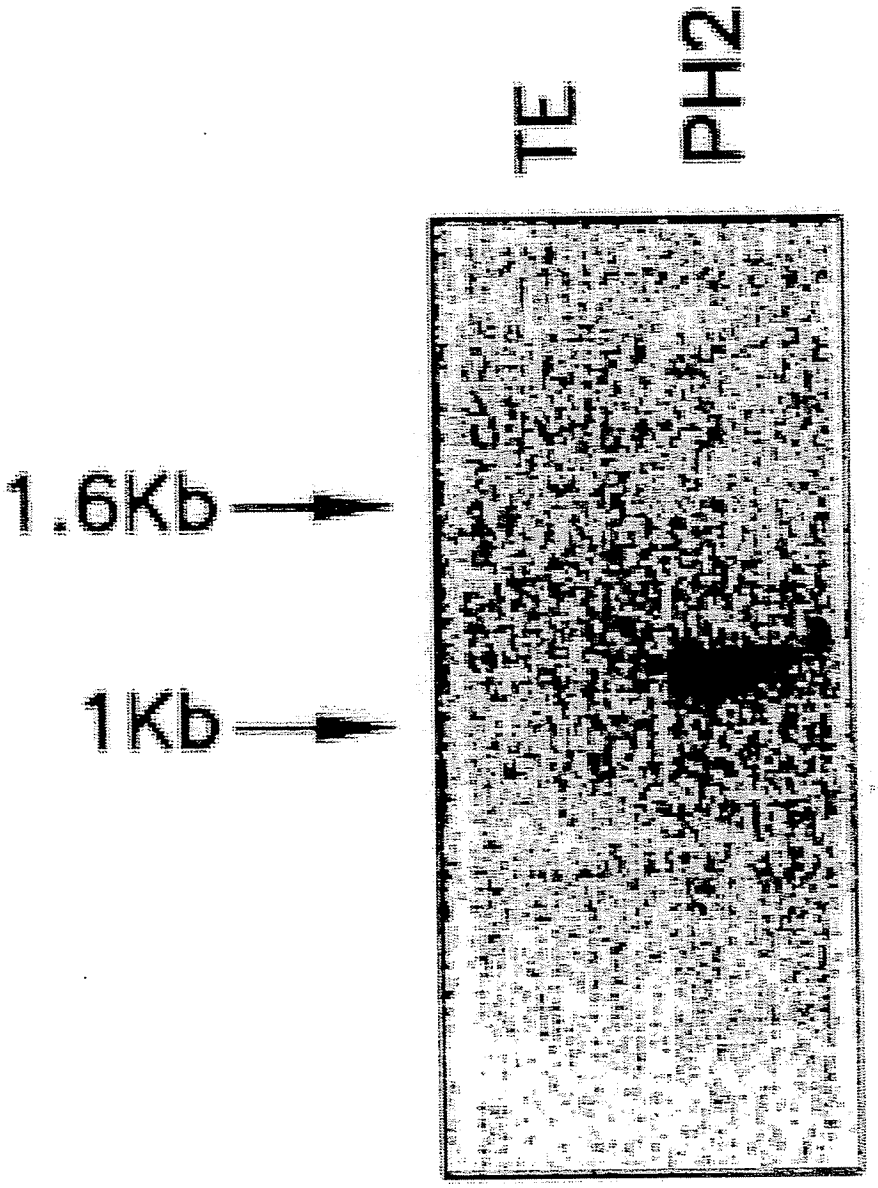


FIG. 6B

METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS

This is a continuation of application Ser. No. 07/467,983, filed Jan. 22, 1990, now abandoned.

BACKGROUND OF THE INVENTION

This invention relates to fertile transgenic plants of the species *Zea mays* (oftentimes referred to herein as maize or corn). The invention further relates to producing transgenic plants via particle bombardment and subsequent selection techniques which have been found to produce fertile transgenic plants.

Genetic engineering of plants, which entails the isolation and manipulation of genetic material (usually in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells, offers considerable promise to modern agriculture and plant breeding. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals, chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques. Once a gene has been identified, cloned, and engineered, it is still necessary to introduce it into a plant of interest in such a manner that the resulting plant is both fertile and capable of passing the gene on to its progeny.

A variety of methods have been developed and are currently available for the transformation of various plants and plant cells with DNA. Generally these plants have been dicotyledonous, and some success has been reported with certain of the monocotyledonous cereals. However, some species have heretofore proven untransformable by any method. Thus, previous to this discovery, no technology had been developed which would permit the production of stably transformed *Zea mays* plants in which the transforming DNA is heritable thereof. This failure in the art is well documented in the literature and has been discussed in a number of recent reviews (Potrykus, 1989; Weising et al., 1988; Cocking et al., 1987).

European Patent Publns. 270,356 (McCabe et al.) and 275,069 (Arntzen et al.) describe the introduction of DNA into maize pollen followed by pollination of maize ears and formation of seeds. The plants germinated from these seeds are alleged to contain the introduced DNA, but there is no suggestion that the introduced DNA was heritable, as has been accomplished in the present invention. Only if the DNA introduced into the corn is heritable can the corn be used in breeding programs as required for successful commercialization of transgenic corn.

Graves et al. (1986) claims Agrobacterium-mediated transformation of *Zea mays* seedlings. The alleged evidence was based upon assays known to produce incorrect results.

Despite extensive efforts to produce fertile transformed corn plants which transmit the transforming DNA to progeny, there have been no reported successes. Many previous failures have been based upon gene transfer to maize protoplasts, oftentimes derived from callus, liquid suspension culture cells, or other maize cells using a variety of transformation techniques. Although several of the techniques have resulted in successful transformation of corn cells, the resulting cells either could not be regenerated into corn plants or the corn plants produced were sterile (Rhodes et al. 1988). Thus, while maize protoplasts and some other cells

have previously been transformed, the resulting transformants could not be regenerated into fertile transgenic plants.

On the other hand, it has been known that at least certain corn callus can be regenerated to form mature plants in a rather straightforward fashion and that the resulting plants were often fertile. However, no stable transformation of maize callus was ever achieved, i.e. there were no techniques developed which would permit a successful stable transformation of a regenerable callus. An example of a maize callus transformation technique which has been tried is the use of Agrobacterium mediated transfer.

The art was thus faced with a dilemma. While it was known that corn protoplast and suspension culture cells could be transformed, no techniques were available which would regenerate the transformed protoplast into a fertile plant. While it was known that corn callus could be regenerated into a fertile plant, there were no techniques known which could transform the callus, particularly while not destroying the ability of the callus both to regenerate and to form fertile plants.

Recently, a new transformation technique has been created based upon the bombardment of intact cells and tissues with DNA-coated microprojectiles. The technique, disclosed in Sanford et al. (1987) as well as in EPO Patent Publication 331,855 of J. C. Sanford et al. based upon U.S. Ser. No. 161,807, filed Feb. 29, 1988, has been shown effective at producing transient gene expression in some plant cells and tissues including those from onion, maize (Klein et al. 1988a), tobacco, rice, wheat, and soybean, and stable expression has been obtained in tobacco and soybeans. In fact, stable expression has been obtained by bombardment of suspension cultures of *Zea mays* Black Mexican Sweet (Klein et al. 1989) which cultures are, however, non-regenerable suspension culture cells, not the callus culture cells used in the process of the present invention.

No protocols have been published describing the introduction of DNA by a bombardment technique into cultures of regenerable maize cells of any type. No stable expression of a gene has been reported by means of bombardment of corn callus followed by regeneration of fertile plants and no regenerable fertile corn has resulted from DNA-coated microprojectile bombardment of the suspension cultures. Thus, the art has failed to produce fertile transformed corn plants heretofore.

A further stumbling block to the successful production of fertile transgenic maize plants has been in selecting those few transformants in such a manner that neither the regeneration capacity nor the fertility of the regenerated transformant are destroyed. Due to the generally low level of transformants produced by a transformation technique, the need for selection of the transformants is self-evident. However, selection generally entails the use of some toxic agent, e.g. herbicide or antibiotic, which can effect either the regenerability or the resultant plant fertility.

It is thus an object of the present invention to produce fertile, stably transgenic, *Zea mays* plants and seeds which transmit the introduced gene to progeny. It is a further object to produce such stably transgenic plants and seeds by a particle bombardment and selection process which results in a high level of viability for a few transformed cells. It is a further object to produce fertile stably transgenic plants of other graminaceous cereals besides maize.

REFERENCES CITED

Armstrong, C. L., et al. (1985) J Planta 164:207–214

Callis, J., et al. (1987) Genes & Develop 1:1183–1200

M. Bevan et al., Nuc. Acids Res., 11, 369 (1983)
Chu, C. C., et al. (1975) Sci Sin (Peking) 18:659–668
Cocking, F., et al. (1987) Science 236:1259–1262
DeWet et al. (1985) Proc Natl Sci U.S.A. 82:7870–7873
Freeling, J. C., et al. (1976) Maydica XXI:97–112
Graves, A., et al. (1986) Plant Mol Biol 7:43–50
Green, C., et al. (1975) Crop Sci 15:417–421
Green, C. E., (1982) Plant Tissue Culture, A Fujiwara ed. Maruzen, Tokyo, Japan pp 107–8
Green, C., et al. (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 367–372
Gritz, L., et al. (1983) Gene 25:179–188
Guilley, H., et al. (1982) Cell 30:763–773
Jefferson, R., et al. (1987) EMBO J 6:3901–3907)
Kamo, K., et al. (1985) Bot Gaz 146:327–334
Klein, T., et al. (1989) Plant Physiol 91:440–444
Klein, T., et al. (1988a) Proc Natl Acad Sci U.S.A. 85:4305–9
Klein, T., et al. (1988b) Bio/Technology 6:559–563
Lu, C., et al. (1982) Theor Appl Genet 62:109–112
McCabe, D., et al. (1988) Bio/Technology 6:923–926
Murashige, T., et al. (1962) Physiol Plant 15:473–497
Neuffer, M., (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 19–30
Phillips, R., et al. (1988) Corn and Corn Improvement, 3rd ed., Agronomy Soc Amer, pp 345–387
Potrykus, I. (1989) Trends in Biotechnology 7:269–273
Rhodes, C. A., et al. (1988) Science 240:204–7
Sambrook, J., et al (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press
Sanford, et al. (1987) J Part Sci & Techn 5:27–37
Weising, K., et al., (1988) Ann Rev of Genetics 22:421–478
Yanisch-Perron, L, et al. (1985) Gene 33:109–119

SUMMARY OF THE INVENTION

The present invention relates to fertile transgenic *Zea mays* plants containing heterologous DNA, preferably chromosomally integrated heterologous DNA, which is heritable by progeny thereof.

The invention further relates to all products derived from transgenic *Zea mays* plants, plant cells, plant parts, and seeds.

The invention further relates to transgenic *Zea mays* seeds stably containing heterologous DNA and progeny which inherit the heterologous DNA.

The invention further relates to a process for producing fertile transgenic *Zea mays* plants containing heterologous DNA. The process is based upon microprojectile bombardment, selection, and plant regeneration techniques.

The invention further relates to a process for producing fertile transformed plants of graminaceous plants other than *Zea mays* which have not been reliably transformed by traditional methods such as electroporation, Agrobacterium, injection, and previous ballistic techniques.

The invention further relates to regenerated fertile mature maize plants from transformed embryogenic tissue, transgenic seeds produced therefrom, and R1 and subsequent generations.

In preferred embodiments, this invention produces the fertile transgenic plants by means of a DNA-coated microprojectile bombardment of clumps of friable embryogenic callus, followed by a controlled regimen for selection of the transformed callus lines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1A** shows a map of plasmid vector pHYGI1 utilized in Example I. FIG. **1B** shows the relevant part of pHYGI1 encompassing the HPT coding sequence and associated regulatory elements. The base pair numbers start from the 5' nucleotide in the recognition sequence for the indicated restriction enzymes, beginning with the EcoRI site at the 5' end of the CaMV 35S promoter.

FIG. **2** shows a map of plasmid vector pBII221 utilized in Example I.

FIG. **3** is a Southern blot of DNA isolated from the PH1 callus line and an untransformed control callus line.

FIG. **4** is a Southern blot of leaf DNA isolated from Ro plants regenerated from PH1 and untransformed callus.

FIG. **5** is a Southern blot of leaf DNA isolated from R1 progeny of PH1 Ro plants and untransformed Ro plants.

FIG. **6** is a Southern blot of DNA isolated from the PH2 callus line and an untransformed control callus line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the production of fertile transgenic plants and seeds of the species *Zea mays* and to the plants, plant tissues, and seeds derived from such transgenic plants, as well as the subsequent progeny and products derived therefrom. The transgenic plants produced herein include all plants of this species, including field corn, popcorn, sweet corn, flint corn and dent corn.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant which contains heterologous DNA that was introduced into plant material by a process of genetic engineering, or which was initially introduced into a plant species by such a process and was subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions.

By "heritable" is meant that the DNA is capable of transmission through a complete sexual cycle of a plant, i.e. passed from one plant through its gametes to its progeny plants in the same manner as occurs in normal corn.

The transgenic plants of this invention may be produced by (i) establishing friable embryogenic callus from the plant to be transformed, (ii) transforming said cell line by a microprojectile bombardment technique, (iii) controllably identifying or selecting transformed cells, and (iv) regenerating fertile transgenic plants from the transformed cells. Some of the plants of this invention may be produced from the transgenic seed produced from the fertile transgenic plants using conventional crossbreeding techniques to develop commercial hybrid seed containing heterologous DNA.

I. Plant Lines and Tissue Cultures

The cells which have been found useful to produce the fertile transgenic maize plants herein are those callus cells which are regenerable, both before and after undergoing a selection regimen as detailed further below. Generally, these cells will be derived from meristematic tissue which contain cells which have not yet terminally differentiated. Such tissue in graminaceous cereals in general and in maize, in

particular, comprise tissues found in juvenile leaf basal regions, immature tassels, immature embryos, and coleoptilar nodes. Preferably, immature embryos are used. Methods of preparing and maintaining callus from such tissue and plant types are well known in the art and details on so doing are available in the literature, c.f. Phillips et al. (1988), the disclosure of which is hereby incorporated by reference.

The specific callus used must be able to regenerate into a fertile plant. The specific regeneration capacity of particular callus is important to the success of the bombardment/selection process used herein because during and following selection, regeneration capacity may decrease significantly. It is therefore important to start with cultures that have as high a degree of regeneration capacity as possible. Callus which is more than about 3 months and up to about 36 months of age has been found to have a sufficiently high level of regenerability and thus is currently preferred. The regenerative capacity of a particular culture may be readily determined by transferring samples thereof to regeneration medium and monitoring the formation of shoots, roots, and plantlets. The relative number of plantlets arising per Petri dish or per gram fresh weight of tissue may be used as a rough quantitative estimate of regeneration capacity. Generally, a culture which will produce at least one plant per gram of callus tissue will be preferred.

While maize callus cultures can be initiated from a number of different plant tissues, the cultures useful herein are preferably derived from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1–3 mm in length. This length generally occurs about 9–14 days after pollination. Under aseptic conditions, the embryos are placed on conventional solid media with the embryo axis down (scutellum up). Callus tissue appears from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" is meant that the tissue is easily dispersed without causing injury to the cells. Tissue with this morphology is then transferred to fresh media and subcultured on a routine basis about every two weeks.

The callus initiation media is solid because callus cannot be readily initiated in liquid medium. The initiation/maintainence media is typically based on the N6 salts of Chu et al. (1975) as described in Armstrong et al. (1985) or the MS salts of Murashige et al. (1962). The basal medium is supplemented with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth. The cultures are generally maintained in the dark, though low light levels may also be used. The level of synthetic hormone 2,4-D, necessary for maintainence and propagation, should be generally about 0.3 to 3.0 mg/l.

Although successful transformation and regeneration has been accomplished herein with friable embryogenic callus, this is not meant to imply that other transformable regenerable cells, tissue, or organs cannot be employed to produce the fertile transgenic plants of this invention. The only actual requirement for the cells which are transformed is that after transformation they must be capable of regeneration of a plant containing the heterologous DNA following the particular selection or screening procedure actually used.

II. DNA Used for Transformation

The heterologous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of a plasmid and contains coding regions of beneficial heterologous DNA with flanking regulatory sequences which serve to promote the expression of the heterologous DNA present in the resultant corn plant. "Heterologous DNA" is used herein to include all synthetically engineered or biologically derived DNA which is introduced into a plant by man by genetic engineering, including but not limited to, non-plant genes, modified genes, synthetic genes, portions of genes, as well as DNA and genes from maize and other plant species.

The compositions of and methods for constructing heterologous DNA for successful transformations of plants is well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the heterologous DNA useful herein. The specific composition of the DNA is not central to the present invention and the invention is not dependent upon the composition of the specific transforming DNA used. Weising et al. (1988), the subject matter of which is incorporated herein by reference, describes suitable DNA components thereof which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, as well as provides suitable references for compositions thereof. Sambrook et al. (1989) provides suitable methods of construction.

Generally the heterologous DNA will be relatively small, i.e. less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the DNA increases.

Suitable heterologous DNA for use herein includes all DNA which will provide for, or enhance, a beneficial feature of the resultant transgenic corn plant. For example, the DNA may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. For example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; bacterial ESPS synthase for resistance to glyphosate herbicide; chitinase or glucan endo-1,3-B-glucosidase for fungicidal properties. Also, the DNA may be introduced to act as a genetic tool to generate mutants and/or assist in the identification, genetic tagging, or isolation of segments of corn DNA. Additional examples may be found in Weising, supra.

The heterologous DNA to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a cotransformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Weising et al, supra. A preferred selectable marker gene is the hygromycin B phosphotransferase (HPT) coding sequence, which may be derived from *E. coli.* Other selectable markers known in the art include aminoglycoside phosphotransferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin, and G418, as well as those genes which code for resistance or tolerance to glyphosate, methotrexate, imidazolinones, sulfonylureas, bromoxynil, dalapon, and the like. Those selectable marker genes which confer herbicide resistance or tolerance are also of commercial utility in the resulting transformed plants.

Reporter genes which encode for easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present or expressed by the recipient

organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g. phenotypic change or enzymatic activity. Examples of such genes are provided in Weising et al, Supra. Preferred genes include the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the beta-glucuronidase gene of the uidA locus of *E. coli*, and the luciferase genes from firefly *Photinus pyralis*.

The regulatory sequences useful herein include any constitutive, inducible, tissue or organ specific, or developmental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al, supra. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *Agrobacterium tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase promoter from corn; light inducible promoters such as, ribulose-biphosphate-carboxylase small subunit gene from a variety of species; and the major chlorophyll a/b binding protein gene promoter; 35S and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present on the DNA. Such elements may or may not be necessary for the function of the DNA, although they can provide a better expression or functioning of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the plant. For example, the maize Adh1S first intron may be placed between the promoter and the coding sequence of a particular heterologous DNA. This intron, when included in a DNA construction, is known to generally increase expression in maize cells of a protein. (Callis et al. 1987) However, sufficient expression for a selectable marker to perform satisfactorily can often be obtained without an intron. (Klein et al. 1989) An example of an alternative suitable intron is the shrunken-1 first intron of *Zea mays*. These other elements must be compatible with the remainder of the DNA constructions.

To determine whether a particular combination of DNA and recipient plant cells are suitable for use herein, the DNA may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the DNA has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (GUS) gene (Jefferson et al. 1987). In the case of the microprojectile bombardment transformation process of the present invention, a suitable time for conducting the assay is about 2–3 days after bombardment. The use of transient assays is particularly important when using DNA components which have not previously been demonstrated or confirmed as compatible with the desired recipient cells.

III. DNA Delivery Process

The DNA can be introduced into the regenerable maize callus cultures via a particle bombardment process. A general description of a suitable particle bombardment instrument is provided in Sanford et al. (1987), the disclosure of which is incorporated herein by reference. While protocols for the use of the instrument in the bombardment of maize non-regenerable suspension culture cells are described in Klein et al. (1988a, 1988b, and 1989), no protocols have been published for the bombardment of callus cultures or regenerable maize cells.

In a microprojectile bombardment process, also referred to as a biolistic process, the transport of the DNA into the callus is mediated by very small particles of a biologically inert material. When the inert particles are coated with DNA and accelerated to a suitable velocity, one or more of the particles is able to enter into one or more of the cells where the DNA is released from the particle and expressed within the cell. While some of the cells are fatally damaged by the bombardment process, some of the recipient cells do survive, stably retain the introduced DNA, and express it.

The particles, called microprojectiles, are generally of a high density material such as tungsten or gold. They are coated with the DNA of interest. The microprojectiles are then placed onto the surface of a macroprojectile which serves to transfer the motive force from a suitable energy source to the microprojectiles. After the macroprojectile and the microprojectiles are accelerated to the proper velocity, they contact a blocking device which prevents the macroprojectile from continuing its forward path but allows the DNA-coated microprojectiles to continue on and impact the recipient callus cells. Suitable such instruments may use a variety of motive forces such as gunpowder or shock waves from an electric arc discharge (J. C. Sanford et al., *J. Particle Science and Technology*, 5, 27 (1987) et al. 1988). An instrument in which gunpowder is the motive force is currently preferred and such is described and further explained in Sanford et al. (1987), the disclosure of which is incorporated herein by reference.

A protocol for the use of the gunpowder instrument is provided in Klein et al. (1988a, b) and involves two major steps. First, tungsten microprojectiles are mixed with the DNA, calcium chloride, and spermidine free base in a specified order in an aqueous solution. The concentrations of the various components may be varied as taught. The currently preferred procedure entails exactly the procedure of Klein et al. (1988b) except for doubling the stated optimum DNA concentration. Secondly, in the actual bombardment, the distance of the recipient cells from the end of the barrel as well as the vacuum in the sample chamber. The currently preferred procedure for bombarding the callus entails exactly the procedure of Klein et al. (1988b) with the recipient tissue positioned 5 cm below the stopping plate tray.

The callus cultures useful herein for generation of transgenic plants should generally be about 3 months to 3 years old, preferably about 3 to 18 months old. Callus used for bombardment should generally be about midway between transfer periods and thus past any "lag" phase that might be associated with a transfer to a new media, but also before reaching any "stationary" phase associated with a long time on the same plate.

The specific tissue subjected to the bombardment process is preferably taken about 7–10 days after subculture, though this is not believed critical. The tissue should generally be used in the form of pieces of about 30 to 80, preferably about 40 to 60, mg. The clumps are placed on a petri dish or other surface and arranged in essentially any manner, recognizing that (i) the space in the center of the dish will receive the heaviest concentration of metal-DNA particles and the tissue located there is likely to suffer damage during bombardment and (ii) the number of particles reaching a cell will decrease (probably exponentially) with increasing distance of the cell from the center of the blast so that cells far from the center of the dish are not likely to be bombarded and transformed.

A mesh screen, preferably of metal, may be laid on the dish to prevent splashing or ejection of the tissue. The tissue may be bombarded one or more times with the DNA-coated metal particles.

IV. Selection Process

Once the calli have been bombarded with the DNA and the DNA has penetrated some of the cells, it is necessary to identify and select those cells which both contain the heterologous DNA and still retain sufficient regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively and preferably, when a selectable marker gene has been transmitted along with or as part of the heterologous DNA, those cells of the callus which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells while simultaneously inhibiting the growth of the non-transformed cells. The situation is complicated by the fact that the vitality of individual cells in a population is often highly dependent on the vitality of neighboring cells. Also, the selection conditions must not be so severe that the plant regeneration capacity of the callus cells and the fertility of the resulting plant are precluded. Thus the effects of the selection agent on cell viability and morphology should be evaluated. This may be accomplished by experimentally producing a growth inhibition curve for the given selective agent and tissue being transformed beforehand. This will establish the concentration range which will inhibit growth.

When a selectable marker gene has been used, the callus clumps may be either allowed to recover from the bombardment on non-selective media or, preferably, directly transferred to media containing that agent.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably the concentration of the agent is initially such that about a 5–40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells to preferentially grow and divide while inhibiting untransformed cells, but not to the extent that growth of the transformed cells is prevented. Once the few individual transformed cells have grown sufficiently the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of non-transformed cells habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative maize transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells. The callus may also be cultured on non-selective media at various times during the overall selection procedure.

Once a callus sector is identified as a putative transformant, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e. Southern blot or polymerase chain reaction (PCR) or the like.

V. Regeneration of Plants and Production of Seed

Cell lines which have been shown to be transformed must then be regenerated into plants and the fertility of the resultant plants determined. Transformed lines which test positive by genotypic and/or phenotypic analysis are then placed on a media which promotes tissue differentiation and plant regeneration. Regeneration may be carried out in accordance with standard procedures well known in the art. The procedures commonly entail reducing the level of auxin which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. One example of such a regeneration procedure is described in Green et al. (1981). The plants are grown to maturity in a growth room or greenhouse and appropriate sexual crosses and selfs are made as described by Neuffer (1981).

Regeneration, while important to the present invention, may be performed in any conventional manner. If a selectable marker has been transformed into the cells, the selection agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are transformed. Since regeneration techniques are well known and not critical to the present invention, any technique which accomplishes the regeneration and produces fertile plants may be used.

VI. Analysis of R1 Progeny

The plants regenerated from the transformed callus are referred to as the R0 generation or R0 plants. The seeds produced by various sexual crosses of the R0 generation plants are referred to as R1 progeny or the R1 generation. When R1 seeds are germinated, the resulting plants are also referred to as the R1 generation.

To confirm the successful transmission and inheritance of the heterologous DNA in the sexual crosses described above, the R1 generation should be analyzed to confirm the presence of the transforming DNA. The analysis may be performed in any of the manners such as were disclosed above for analyzing the bombarded callus for evidence of transformation, taking into account the fact that plants and plant parts are being used in place of the callus.

VII. Breeding of Genetically Engineered Commercial Hybrid Seed

Generally, the commercial value of the transformed corn produced herein will be greatest if the heterologous DNA can be incorporated into many different hybrid combinations. A farmer typically grows several varieties of hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based

upon his physical location since hybrids adapted to one part of the corn belt are generally not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the heterologous DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable heterologous DNA. This may conveniently be done by breeding programs in which a conversion process (backcrossing) is performed by crossing the initial transgenic fertile plant to normal elite inbred lines and then crossing the progeny back to the normal parent. The progeny from this cross will segregate such that some of the plants will carry the heterologous DNA whereas some will not. The plants that do carry the DNA are then crossed again to the normal plant resulting in progeny which segregate once more. This crossing is repeated until the original normal parent has been converted to a genetically engineered line containing the heterologous DNA and also possessing all other important attributes originally found in the parent. A separate backcrossing program will be used for every elite line that is to be converted to a genetically engineered elite line. It may be necessary for both parents of a hybrid seed corn to be homozygous for the heterologous DNA. Corn breeding and the techniques and skills required to transfer genes from one line or variety to another are well-known to those skilled in the art. Thus introducing heterologous DNA into lines or varieties which do not generate the appropriate calli can be readily accomplished by these breeding procedures.

VIII. Uses of Transgenic Plants

The transgenic plants produced herein are expected to be useful for a variety of commercial and research purposes. Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g. agronomic traits such as pest resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g. improved nutritive content in human food or animal feed), or beneficial to the food processor (e.g. improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods. However, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silage or for ornamental purposes (e.g. Indian corn). Often chemical constituents (e.g. oils or starches) of corn and other crops are extracted for food or industrial use and transgenic plants may be created which have enhanced or modified levels of such components. The plants may also be used for seed production for a variety of purposes.

Transgenic plants may also find use in the commercial manufacture of proteins or other molecules encoded by the heterologous DNA contained therein, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules, or for other purposes (e.g. for research).

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded by the heterologous DNA may be transferred, e.g. from corn cells to cells of other species e.g. by protoplast fusion.

The transgenic plants may have many uses in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. The methods of the invention may also be used to create plants having unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties.

The following non-limiting examples are illustrative of the present invention. They are presented to better explain the general procedures which were used to prepare the fertile *Zea mays* plants of this invention which stably express the heterologous DNA and which transmit that DNA to progeny. All parts and percents are by weight unless otherwise specified. It must be recognized that a specific transformation event is a function of the amount of material subjected to the transformation procedure. Thus when individual situations arise in which the procedures described herein do not produce a transformed product, repetition of the procedures will be required.

EXAMPLE I

Fertile transgenic *Zea mays* plants which contain heterologous DNA which is heritable were prepared as follows:

I. Initiation and Maintenance of Maize Cell Cultures which Retain Plant Regeneration Capacity

Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred line A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient agar initiation/maintenance media with the root/shoot axis exposed to the medium. Initiation/maintenance media (hereinafter referred to as F medium) consisted of N6 basal media (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc. San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

The immature embryos were incubated at 26° C. in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh media. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell culture named AB12 used in this example was such a culture and had been initiated about 1 year before bombardment.

II. Plasmids—pCHN1-1, pHYGI1, pBII221, and pLUC-1

The plasmids pCHN1-1, pHYGI1, and pLUC-1 were constructed in the vector pBS+ (Stratagene, Inc., San Diego, Calif.), a 3.2 Kb circular plasmid, using standard recombinant DNA techniques. pCHN1-1 contains the hygromycin B phosphotransferase (HPT) coding sequence from *E. coli* (Gritz et al. 1983) flanked at the 3' end by the nopaline synthase (nos) polyadenylation sequence of *Agrobacterium tumefaciens* (M. Bevan et al., Nuc. Acids Res., 11, 369, 1983). Expression is driven by the cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982), located

upstream from the hygromycin coding sequence. The plasmid pHYGI1 was constructed by inserting the 553 bp Bcl-BamHI fragment containing the maize Adh1S first intron (Callis et al. 1987) between the CaMV 35S promoter and the hygromycin coding sequence of pCHN1-1. A map of pHYGI1 is provided as FIG. 1.

pBII221 contains the *E. coli* B-glucuronidase coding sequence flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. The plasmid was constructed by inserting the maize AdhIS first intron between the 35S promoter and the coding sequence of pBI221 (Jefferson et al. 1987). A map of pBII221 is provided as FIG. 2.

pLUC-1 contains the firefly luciferase coding sequence (DeWet et al. 1987) flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. This plasmid was used solely as a negative control.

Plasmids were introduced into the embryogenic callus culture AB12 by microprojectile bombardment.

III. DNA Delivery Process

The embryogenic maize callus line AB12 was subcultured 7 to 12 d prior to microprojectile bombardment. AB12 was prepared for bombardment as follows. Five clumps of callus, each approximately 50 mg in wet weight were arranged in a cross pattern in the center of a sterile 60×15 mm petri plate (Falcon 1007). Plates were stored in a closed container with moist paper towels throughout the bombardment process. Twenty six plates were prepared.

Plasmids were coated onto M-10 tungsten particles (Biolistics) exactly as described by Klein, et al (1988b) except that, (i) twice the recommended quantity of DNA was used, (ii) the DNA precipitation onto the particles was performed at 0° C., and (iii) the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process.

All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. When two plasmids were used simultaneously, each was present in an amount of 2.5 ul. One tube contained only plasmid pBII221; two tubes contained both plasmids pHYGI1 and pBII221; two tubes contained both plasmids pCHN1-1 and pBII221; and one tube contained only plasmid pLUC-1.

All tubes were incubated on ice for 10 min., pelletized by centrifugation in an Eppendorf centrifuge at room temperature for 5 seconds, and 25 ul of the supernatant was discarded. The tubes were stored on ice throughout the bombardment process. Each preparation was used for no more than 5 bombardments.

Macroprojectiles and stopping plates were obtained from Biolistics, Inc. (Ithaca, N.Y.). They were sterilized as described by the supplier. The microprojectile bombardment instrument was obtained from Biolistics, Inc.

The sample plate tray was positioned at the position 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate in the slot below the barrel. Plates of callus tissue prepared as described above were centered on the sample plate tray and the petri dish lid removed. A 7×7 cm square rigid wire mesh with 3×3 mm mesh and made of galvanized steel was placed over the open dish in order to retain the tissue during the bombardment. Tungsten/DNA preparations were sonicated as described by Biolistics, Inc. and 2.5 ul was pipetted onto the top of the macroprojectiles. The instrument was operated as described by the manufacturer. The following bombardments were performed:

2 × pBII221 prep	To determine transient expression frequency
10 × pHYGI1/pBII221	As a potential positive treatment for transformation
10 × pCHN1-1/pBII221	As a potential positive treatment for transformation
4 × pLUC-1	Negative control treatment

The two plates of callus bombarded with pBII221 were transferred plate for plate to F medium (with no hygromycin) and the callus cultured at 26° C. in the dark. After 2 d this callus was then transferred plate for plate into 35×10 mm petri plates (Falcon 1008) containing 2 ml of GUS assay buffer which consists of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (Research Organics), 100 mM sodium phosphate pH 7.0, 5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM EDTA, and 0.06% Triton X-100. These were incubated at 37° C. for 3 d after which the number of blue cells was counted giving 291 and 477 transient GUS expressing cells in the two plates, suggesting that the DNA delivery process had also occurred with the other bombarded plates. These plates were discarded after counting since the GUS assay is destructive.

IV. Selection Process

Hygromycin B (Calbiochem) was incorporated into the medium by addition of the appropriate volume of filter sterilized 100 mg/ml Hygromycin B in water when the media had cooled to 45° C. prior to pouring plates.

Immediately after all samples had been bombarded, callus from all of the plates treated with pHYGI1/pBII221, pCHN1-1/pBII- 221 and three of the plates treated with pLUC-1 were transferred plate for plate onto F medium containing 15 mg/l hygromycin B, (five pieces of callus per plate). These are referred to as round 1 selection plates. Callus from the fourth plate treated with pLUC-1 was transferred to F medium without hygromycin. This tissue was subcultured every 2–3 weeks onto nonselective medium and is referred to as unselected control callus.

After two weeks of selection, tissue appeared essentially identical on both selective and nonselective media. All callus from eight plates from each of the pHYGI1/pBII221 and pCHN1-1/pBII- 221 treatments and two plates of the control callus on selective media were transferred from round 1 selection plates to round 2 selection plates that contained 60 mg/l hygromycin. The round 2 selection plates each contained ten 30 mg pieces of callus per plate, resulting in an expansion of the total number of plates.

The remaining tissue on selective media, two plates each of pHYGI1/pBII221 and pCHN1-1/pBII221 treated tissue and one of control callus, were placed in GUS assay buffer at 37° C. to determine whether blue clusters of cells were observable at two weeks post-bombardment. After 6 days in assay buffer this tissue was scored for GUS expression.

TREATMENT	REPLICATE	OBSERVATIONS
pLUC-1		no blue cells
PHYGI1/PBII221	plate 1	11 single cells 1 four cell cluster
	plate 2	5 single cells
pCHN1-1/pBII221	plate 1	1 single cell 2 two cell clusters
	plate 2	5 single cells 1 two cell cluster 2 clusters of 8–10 cells

After 21 days on the round 2 selection plates, all viable portions of the material were transferred to round 3 selection

plates containing 60 mg/l hygromycin. The round 2 selection plates, containing only tissue that was apparently dead, were reserved. Both round 2 and 3 selection plates were observed periodically for viable proliferating sectors.

After 35 d on round 3 selection plates both the round 2 and round 3 sets of selection plates were checked for viable sectors of callus. Two such sectors were observed proliferating from a background of dead tissue on plates treated with pHYGI1/pBII221. The first sector named 3AA was from the round 3 group of plates and the second sector named 6L was from the round 2 group of plates. Both lines were then transferred to F medium without hygromycin.

After 19 days on F medium without hygromycin the line 3AA grew very little whereas the line 6L grew rapidly. Both were transferred again to F medium for 9 d. The lines 3AA and 6L were then transferred to F medium containing 15 mg/l hygromycin for 14 days. At this point, line 3AA was observed to be of very poor quality and slow growing. The line 6L however grew rapidly on F medium with 15 mg.l hygromycin. In preparation for an inhibition study of the line 6 L on hygromycin, the line was then subcultured to F medium without hygromycin.

After 10 days on F medium an inhibition study of the line 6L was initiated. Callus of 6L was transferred onto F medium containing 0, 10, 30, 100, and 250 mg/l hygromycin B. Five plates of callus were prepared for each concentration and each plate contained ten approximately 50 mg pieces of callus. One plate of unselected control tissue was prepared for each concentration of hygromycin.

It was found that the line 6L was capable of sustained growth over 9 subcultures on 0, 10, 30, 100, and 250 mg/l hygromycin. The name of the line 6L was changed at this time from 6L to PH1 (Positive Hygromycin transformant 1).

Additional sectors were recovered at various time points from the round 2 and 3 selection plates. None of these were able to grow in the presence of hygromycin for multiple rounds, i.e. two or three subcultures.

V. Confirmation of Transformed Callus

To show that the PH1 callus had acquired the hygromycin resistance gene, a Southern blot of PH1 callus was prepared as follows: DNA was isolated from PH1 and unselected control calli by freezing 2 g of callus in liquid nitrogen and grinding it to a fine powder which was transferred to a 30 ml Oak Ridge tube containing 6 ml extraction buffer (7M urea, 250 mM NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1% sarcosine). To this was added 7 ml of phenol:chloroform 1:1, the tubes shaken and incubated at 37° C. 15 min. Samples were centrifuged at 8K for 10 min. at 4° C. The supernatant was pipetted through miracloth (Calbiochem 475855) into a disposable 15 ml tube (American Scientific Products, C3920-15A) containing 1 ml 4.4M ammonium acetate, pH 5.2. Isopropanol, 6 ml, was added, the tubes shaken, and the samples incubated at –20° C. for 15 min. The DNA was pelleted in a Beckman TJ-6 centrifuge at the maximum speed for 5 min. at 4° C. The supernatant was discarded and the pellet was dissolved in 500 ul TE-10 (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) 15 min. at room temperature. The samples were transferred to a 1.5 ml Eppendorf tube and 100 ul 4.4M ammonium acetate, pH 5.2 and 700 ul isopropanol were added. This was incubated at –20° C. for 15 min. and the DNA pelleted 5 min. in an Eppendorf microcentrifuge (12,000 rpm). The pellet was washed with 70% ethanol, dried, and resuspended in TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The isolated DNA (10 ug) was digested with BamHI (NEB) and electrophoresed in a 0.8% w/v agarose gel at 15 V for 16 hrs in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The DNA within the gel was then depurinated by soaking the gel twice in 0.25M HCl for 15 min., denatured and cleaved by soaking the gel twice in 0.5M NaOH/1.0M NaCl 15 min., and neutralized by soaking the gel twice in 0.5M Tris pH 7.4/3M NaCl 30 min. DNA was then blotted onto a Nytran membrane (Shleicher & Shuell) by capillary transfer overnight in 6×SSC (20×SSC, 3M NaCl, 0.3M sodium citrate pH 7.0). The membrane was baked at 80° C. for 2 hrs under vacuum. Prehybridization treatment of the membrane was done in 6×SSC, 10× Denhardt's solution, 1% SDS, 50 ug/ml denatured salmon sperm DNA using 0.25 ml prehybridization solution per cm^2 of membrane. Prehybridization was carried out at 42° C. overnight.

A 32P labelled probe was prepared by random primer labelling with an Oligo Labelling Kit (Pharmacia) as per the suppliers instructions with 32P-dCTP (ICN Radiochemicals). The template DNA used was the 1055 bp BamHI fragment of pHYGI1, which is the HPT coding sequence. The fragment was gel purified and cut again with PstI (NEB) before labelling.

The hybridization was performed in 50% formamide, 6×SSC, 1% SDS, 50 ug/ml denatured salmon sperm DNA (Sigma), 0.05% sodium pyrophosphate and all of the isopropanol precipitated heat denatured probe (107 CPM/50ug template). The hybridization was carried out at 42° C. overnight.

The membrane was washed twice in 50 ml 6×SSC, 0.1% SDS 5 min. at room temperature with shaking, then twice in 500 ml 6×SSC, 0.1% SDS 15 min. at room temperature, then twice in 500 ml 1×SSC, 1% SDS 30 min. at 42° C., and finally in 500 ml 0.1×SSC 1% SDS 60 min. at 65° C. Membranes were exposed to Kodak X-OMAT AR film in an X-OMATIC cassette with intensifying screens. As shown in FIG. **3**, a band was observed for PH1 callus at the expected position of 1.05 Kb, indicating that the HPT coding sequence was present. No band was observed for control callus.

VI. Plant Regeneration and Production of Seed

PH1 callus was transferred directly from all of the concentrations of hygromycin used in the inhibition study to RM5 medium which consists of MS basal salts (Murashige et al. 1962) supplemented with thiamine HCl 0.5 mg/l, 2,4-D 0.75 mg/l, sucrose 50 g/l, asparagine 150 mg/l, and Gelrite 2.5 g/l (Kelco Inc. San Diego).

After 14 days on RM5 medium the majority of PH1 and negative control callus was transferred to R5 medium which is the same as RM5 medium, except that 2,4-D is omitted. These were cultured in the dark for 7 d at 26° C. and transferred to a light regime of 14 hours light and 10 hours dark for 14 d at 26° C. At this point, plantlets that had formed were transferred to one quart canning jars (Ball) containing 100 ml of R5 medium. Plants were transferred from jars to vermiculite after 14 and 21 d. Plants were grown in vermiculite for 7 or 8 days before transplanting into soil and grown to maturity. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the Ro tissue, a Southern blot was performed as previously described on leaf DNA from three randomly chosen Ro plants of PH1. As shown in FIG. **4**, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred lines A188, B73 and Oh43. Seed was harvested 45 days post-pollination and

allowed to dry further 1–2 weeks. Seed set varied from 0 to 40 seeds per ear when PH1 was the female parent and from 0 to 32 seeds per ear when PH1 was the male parent.

VII. Analysis of the R1 Progeny

The presence of the hygromycin resistance trait was evaluated by a root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears.

(A) Root Elongation Bioassay

Seed was sterilized in a 1:1 dilution of commercial bleach in water plus alconox 0.1% for 20 min. in 125 ml Erlenmeyer flasks and rinsed 3 times in sterile water and imbibed overnight in sterile water containing 50 mg/ml captan by shaking at 150 rpm.

After imbibition, the solution was decanted from the flasks and the seed transferred to flow boxes (Flow Laboratories) containing 3 sheets of H_2O saturated germination paper. A fourth sheet of water saturated germination paper was placed on top of the seed. Seed was allowed to germinate 4 d.

After the seed had germinated, approximately 1 cm of the primary root tip was excised from each seedling and plated on MS salts, 20 g/l sucrose, 50 mg/l hygromycin, 0.25% Gelrite, and incubated in the dark at 26° C. for 4 d.

Roots were evaluated for the presence or absence of abundant root hairs and root branches. Roots were classified as transgenic (hygromycin resistant) if they had root hairs and root branches, and untransformed (hygromycin sensitive) if they had limited numbers of branches. The results are shown in Table 1.

(B) Etiolated Leaf Bioassay

After the root tips were excised as described above, the seedlings of one PH1 ear and one control ear were transferred to moist vermiculite and grown in the dark for 5 d. At this point 1 mm sections were cut from the tip of the coleoptile, surface sterilized 10 seconds, and plated on MS basal salts, 20 g/l sucrose, 2.5 g/l Gelrite with either 0 (control) or 100 mg/l hygromycin and incubated in the dark at 26° C. for 18 hr. Each plate contained duplicate sections of each shoot. They were then incubated in a light regimen of 14 hours light 10 hours dark at 26° C. for 48 hr, and rated on a scale of from 0 (all brown) to 6 (all green) for the percent of green color in the leaf tissue. Shoots were classified as untransformed (hygromycin sensitive) if they had a rating of zero and classified as transformed (hygromycin resistant) if they had a rating of 3 or greater. The results are shown in Table 1.

(C) Southern Blots

Seedling form the bioassays were transplanted to soil and are growing to sexual maturity. DNA was isolated from 0.8 g of leaf tissue after about 3 weeks and probed with the HPT coding sequence as described previously. Plants with a 1.05 Kb band present in the Southern blot were classified as transgenic. As shown in FIG. 5, two out of seven progeny of PH1 plant 3 were transgenic as were three out of eight progeny of PH1 plant 10. The blot results correlated precisely with data from the bioassays, confirming that the heterologous DNA was transmitted through one complete sexual life cycle. All data are summarized in Table 1.

TABLE 1

	ANALYSIS OF PH1 R1 PLANTS						
PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.1	+	ND	+	4.1	–	ND	ND
3.2	–	ND	–	4.2	–	ND	ND
3.3	–	ND	–	4.3	–	ND	ND
3.4	–	ND	–	4.4	–	ND	ND
3.5	–	ND	–	4.5	–	ND	ND
3.6	+	ND	+	4.6	–	ND	ND
3.7	–	ND	–	4.7	–	ND	ND
				2.1	–	ND	–
10.1	+	+	+	1.1	–	–	–
10.2	+	+	+	1.2	–	–	ND
10.3	–	–	ND	1.3	–	–	ND
10.4	–	–	–	1.4	–	–	ND
10.5	–	–	–	1.5	–	–	ND
10.6	–	–	–	1.6	–	–	ND
10.7	–	–	–	1.7	–	–	ND
10.8	ND	+	+	1.8	–	–	ND

KEY: + = transgenic; – = nontransgenic; ND = not done

EXAMPLE II

The procedure of Example I was repeated with minor modifications.

I. Plant Lines and Tissue Cultures

The embryogenic maize callus line, AB12, was used as in Example I. The line had been initiated about 18 months before the actual bombardment occurred.

II. Plasmids

The plasmids pBII221 and pHYGI1 described in Example I were used.

III. DNA Delivery Process

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. One tube contained only plasmid pBII221; two tubes contained only plasmid pHYGI1; and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

2 × pBII221 prep	For transient expression
7 × pHYGI1 prep	Potential positive treatment
3 × TE prep	Negative control treatment

After all the bombardments were performed, the callus from the pBII221 treatments was transferred plate for plate to F medium as five 50 mg pieces. After 2 d the callus was

placed into GUS assay buffer as per Example I. Numbers of transiently expressing cells were counted and found to be 686 and 845 GUS positive cells, suggesting that the particle delivery process had occurred in the other bombarded plates.

IV. Selection of Transformed Callus

After bombardment the callus from the pHYGI1 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate (different from Example I). The same was done for two of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 13 d the callus on round 1 selection plates was indistinguishable from unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin. An approximate five fold expansion of the numbers of plates occurred.

The callus on round 2 selection plates had increased substantially in weight after 23 d, but at this time appeared close to dead. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin. This transfer of all material from round 2 to round 3 selection differs from Example I in which only viable sectors were transferred and the round 2 plates reserved.

At 58 d post-bombardment three live sectors were observed proliferating from the surrounding dead tissue. All three lines were from pHYGI1 treatments and were designated 24C, 56A, and 55A.

After 15 d on maintainance medium, growth of the lines was observed. The line 24C grew well whereas lines 55A and 56A grew more slowly. All three lines were transferred to F medium containing 60 mg/l hygromycin. Unselected control callus from maintenance was plated to F medium having 60 mg/l hygromycin.

After 19 d on 60 mg/l hygromycin the growth of line 24C appeared to be entirely uninhibited, with the control showing approximately 80% of the weight gain of 24C. The line 56A was completely dead, and the line 55A was very close. The lines 24C and 55A were transferred again to F 60 mg/l hygromycin as was the control tissue.

After 23 d on 60 mg/l hygromycin the line 24C again appeared entirely uninhibited. The line 55A was completely dead, as was the negative control callus on its second exposure to to F 60 mg/l hygromycin.

At 88 d post-bombardment, a sector was observed proliferating among the surrounding dead tissue on the round 3 selection plates. The callus was from a plate bombarded with pHYGI1 and was designated 13E. The callus was transferred to F medium and cultured for 19 d. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin. After 14 d of culture, the callus line 13E appeared uninhibited on both levels of hygromycin. The control callus appeared to have about 80% of the weight gain of 13E. The callus lines were transferred to fresh media at the same respective levels of hygromycin.

V. Confirmation of Transformed Callus

A Southern blot was prepared from DNA from the line 24C. As shown in FIG. **6**, a band was observed for the line 24C at the expected size of 1.05 Kb showing that the line 24C contained the HPT coding sequence. No band was observed for DNA from control tissue. The name of the callus line 24C was changed to PH2.

VI. Plant Regeneration and Production of Seed

The line 24C along with unselected control callus were placed onto RM5 medium to regenerate plants as in Example I. After 16 d the callus was transferred to R5 medium as in Example I.

EXAMPLE III

The procedure of Example II was repeated exactly except that different plasmids were used.

The plasmids pBII221 and pHYGI1 described in Example I were used as well as pMS533 which is a plasmid that contains the insecticidal Bacillus thuringiensis endotoxin (BT) gene fused in frame with the neomycin phosphotransferase (NPTII) gene. 5' of the fusion gene are located segments of DNA from the CaMV 35S and nopaline synthase promoters. 3' from the fusion gene are segments of DNA derived from the tomato protease inhibitor I gene and the poly A region of the nopaline synthase gene.

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. Two tubes contained plasmids pHYGI1 and pMS533 and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

9 × pHYGI1/pMS533	Potential positive treatment
2 × TE prep	Control treatment

After bombardment the callus from the pHYGI1/pMS533 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate. The same was done for one of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 12 d the callus on round 1 selection plates appeared to show about 90% of the weight gain of the unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin as ten 30 mg pieces per plate.

After 22 d of selection on round 2 selection plates, the callus appeared completely uninhibited. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin.

At 74 d post-bombardment a single viable sector was observed proliferating from the surrounding necrotic tissue. The callus line was from pHYGI1/pMS533 treated material and was designated 86R. The callus line 86R was transferred to F medium.

After 24 d the callus line 86R had grown substantially. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin.

After 19 d of culture, the callus line 86R appeared to grow rapidly and was uninhibited on both levels of hygromycin. The control callus appeared to have only about 50% of the weight gain of 86R. The callus lines were transferred to fresh

media at the same respective levels of hygromycin to further test the resistance of the callus line 86R to hygromycin.

Comparative Example A

The basic procedures of Examples I–III have been attempted except varying the selection regime or the form of the callus. These other attempts, which are detailed in Table 2 below, were not successful. Since they were not repeated several times, it is not known whether they can be made to work. In all of the procedures, no viable sectors were observed. In the Table, "Sieved" indicates that the callus was passed through an 860 micron sieve before bombardment; the selective agent was hygromycin for each case except when pMXTI1 was the plasmid and methotrexate the selection agent.

TABLE 2

Summary of Comparative Example A

Recip. Tissue	Plasmids	Recov. Period	Round 1 Level	Round 1 Period	Round 2 Level	Round 2 Period
Clumps	pCHN1-1 pBII221	13	60	21	60	81
Clumps	pCHN1-1 pBII221	14	100	22	—	—
Clumps	pHYGI1 pBII221	8	60	19	30	132
Clumps	pCHN1-1 pBII221	0	30	22	60	109
Clumps	pMTXI1 pBII221	8	3	103	—	—
Sieved	pCHN1-1 pBII221	13	—	—	—	—

What is claimed is:

1. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) establishing a regenerable embryogenic callus culture from a *Zea mays* plant to be transformed, (ii) transforming said culture by bombarding it with DNA-coated microprojectiles, (iii) identifying or selecting a transformed cell line, and (iv) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny and imparts herbicide or insect resistance thereto.

2. The process of claim **1** wherein the callus culture subjected to bombardment is in clumps of about 30 to 80 mg per clump.

3. The process of claim **1** wherein said callus is initiated on solid media.

4. The process of claim **1** wherein the DNA comprises a selectable marker gene or a reporter gene.

5. The process of claim **4** wherein said selectable marker gene imparts herbicide resistance to said fertile transgenic *Zea mays* plant.

6. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) establishing a regenerable embryonic callus culture from a *Zea mays* plant to be transformed, (ii) transforming said culture by bombarding it with DNA-coated microprojectiles, (iii) identifying or selecting a transformed cell line, and (iv) regenerating a fertile transgenic *Zea mays* plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, and imparts insect resistance thereto.

7. The process of claim **6** wherein the callus culture subjected to bombardment is in clumps of about 30 to 80 mg per clump.

8. The process of claim **7** wherein said callus is initiated on solid media.

9. The process of claim **6** wherein the DNA comprises a selectable marker gene or a reporter gene.

10. The process of claim **9** wherein said selectable marker gene imparts herbicide resistance to said fertile transgenic *Zea mays* plant.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,538,877
DATED : July 23, 1996
INVENTOR(S) : Lundquist et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

At Col. 22, lines 6-7, please delete "or insect".

Signed and Sealed this

Twenty-sixth Day of May, 1998

Attest:

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

EXHIBIT C

US005538880A

United States Patent [19]

Lundquist et al.

[11] Patent Number: 5,538,880

[45] Date of Patent: * Jul. 23, 1996

[54] **METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS**

[75] Inventors: **Ronald C. Lundquist**, Minnetonka; **David A. Walters**, Bloomington, both of Minn.

[73] Assignee: **DeKalb Genetics Corporation**, St. Paul, Minn.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,538,877.

[21] Appl. No.: **249,458**

[22] Filed: **May 26, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 974,379, Nov. 10, 1992, which is a continuation of Ser. No. 467,983, Jan. 22, 1990, abandoned.

[51] **Int. Cl.**[6] **C12N 15/00**; C12N 15/05; A01H 1/06; A01H 4/00

[52] **U.S. Cl.** **435/172.3**; 435/172.1; 435/240.48; 435/240.49; 935/52; 935/55; 935/67; 935/85; 800/205; 800/235; 800/DIG. 56

[58] **Field of Search** 435/172.3, 172.1, 435/240.4, 240.45, 240.49, 287; 800/205, 235; 935/52, 53, 55, 67, 85

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,370,160	1/1983	Ziemelis	71/117
4,559,302	12/1985	Ingolia	435/172.3
4,581,847	4/1986	Hibberd et al.	47/58
4,665,030	5/1987	Close	435/240
4,666,844	5/1987	Cheng	435/240
4,727,028	2/1988	Santerre et al.	435/240.2
4,806,483	2/1989	Wang	435/240.49
4,940,835	7/1990	Shah et al.	800/205
5,049,500	9/1991	Arnizen et al.	435/172.3

FOREIGN PATENT DOCUMENTS

0126537A2	4/1983	European Pat. Off.	A61K 9/52
0141373A3	5/1985	European Pat. Off.	A01G 7/00
0154204A2	9/1985	European Pat. Off.	C12N 15/00
0160390A2	11/1985	European Pat. Off.	A01H 15/10
0204549A2	10/1986	European Pat. Off.	C12N 15/00
0202668A2	11/1986	European Pat. Off.	C12N 5/02
0242236A1	10/1987	European Pat. Off.	C12N 15/00
0242246A1	11/1987	European Pat. Off.	C12N 15/00
0299552A1	1/1988	European Pat. Off.	C12N 15/00
0262971A2	5/1988	European Pat. Off.	A01H 1/02
0270356A2	6/1988	European Pat. Off.	C12N 15/00
0275069A2	7/1988	European Pat. Off.	C12N 15/00
0280400A2	8/1988	European Pat. Off.	A01C 1/06
0282164A2	9/1988	European Pat. Off.	C12N 5/00
0292435A1	11/1988	European Pat. Off.	C12N 15/00
0289479A2	11/1988	European Pat. Off.	C12N 15/00
0290395A2	11/1988	European Pat. Off.	C12N 15/00
0301749A2	2/1989	European Pat. Off.	C12N 15/00
0334539A2	9/1989	European Pat. Off.	C12N 15/00
0331855A2	9/1989	European Pat. Off.	C12M 3/00
0348348A2	12/1989	European Pat. Off.	A01N 65/00
04421741	4/1991	European Pat. Off.	C12N 15/82
3738874A1	11/1988	Germany	A01H 1/06
8801444	1/1990	Netherlands	C12N 15/87
2159173	11/1985	United Kingdom	C12N 15/00
WO85/01856	5/1985	WIPO	A01B 76/00
WO85/02972	7/1985	WIPO	A01C 1/06
WO87/05629	9/1987	WIPO	C12N 15/00
WO89/04371	5/1989	WIPO	C12N 21/00
WO89/12102	12/1989	WIPO	C12N 15/00
WO90/10691	8/1990	WIPO	C12N 5/00

OTHER PUBLICATIONS

"Bullets" Transform Plant Cells, *Agricell Report*, 9, 5 (Jul. 1987).

"Shotgunning DNA into Cells," *Genetic Engineering News*, (Jul./Aug. 1987).

Ahokes, H. "Electrophoretic transfection of cereal grains with exogenous nucleic acid," Soc. Biochem. Biophys. Microbio. Fen., Biotieteen Paivat (Bioscience Days), Technical University of Helsinki, Espoo, p. 2 (1989).

Armstrong, C. L., et al., "Genetic and cytogenetic variation in plants regenerated from organogenic and friable, embryonic tissue cultures of maize," *Biological Abstracts*, vol. 85, Abstract No. 117662 (1988).

Barker, R. F., et al., "Nucleotide Sequence of the T-DNA Region from the *Agrobacterium tumefaciens* Octopone Ti Plasmid pTi15955," *Plant Mol. Biol., 2,* 335–350 (1983).

Bevan, M., et al., "A Chimaeric Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation," *Nature, 304,* 184–187 (1983).

Bevan, M., et al., "Structure and Transcription of the Nopaline Synthase Gene Region of T-DNA," *Nuc. Acids Res., 11,* 369–385 (1983).

Booy, G., et al., "Attempted Pollen-Mediated Transformation of Maize," *J. Plant Physiol., 135,* 319–324 (1989).

Callis, J., et al., "Introns Increase Gene Expression in Cultures Maize Cells," *Genes and Development, 1,* 1183–1200 (1987).

Cao, J., et al., "Transformation of Rice and Maize using the Biolistic Process," In: *Plant Gene Transfer,* Alan R. Liss, Inc., pp. 21–33 (1990).

Chandler, V. L., et al., "Two Regulatory Genes of the Maize Anthocyanin Pathway are Homologous Isolation of B Utilizing R Genomic Sequences," *The Plant Cell, 1,* 1175–1183 (1989).

Christou, P., et al., "Cotransformation Frequencies of Foreign Genes in Soybean Cell Cultures," *Theor. Appl. Genet., 79,* 337–341 (1990).

(List continued on next page.)

Primary Examiner—Gary Benzion

Attorney, Agent, or Firm—Schwegman, Lundberg, Woessner & Kluth

[57] **ABSTRACT**

Fertile transgenic *Zea mays* (corn) plants which stably express heterologous DNA which is heritable are disclosed along with a process for producing said plants. The process comprises the microjectile bombardment of friable embryogenic callus from the plant to be transformed. The process may be applicable to other graminaceous cereal plants which have not proven stably transformable by other techniques.

18 Claims, 10 Drawing Sheets

OTHER PUBLICATIONS

Christou, P., et al., "Stable Transformation of Soybean Callus by DNA–Coated Gold Particles," *Plant Physiol., 87,* 671–674 (1988).

Cocking, F., et al., "Gene Transfer in Cereals," *Science, 236,* 1259–1262 (1987).

Creissen, G., et al., "*Agrobacterium*– and Microprojectile–Mediated Viral DNA Delivery into Barley Microspore Derived–Cultures," *Plant Cell Rep., 8,* 680–683 (Apr. 1990).

Crossway, A., et al., "Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts," *Mol. Gen. Genet., 202,* 179–185 (1986).

De Block, M., et al., "Engineering herbicide resistance on plants by expression of a detoxifying enzyme," *EMBO J., 6,* 2513–2518 (1987).

De Greef, W., et al., "Evaluation of herbicide resistance in transgenic crops under field conditions," *Bio/Technol., 7,* 61–64 (1989).

Dekeyser, R. A., et al., "Evaluation of Selectable Markers for Rice Transformation," *Plant Physiol., 90,* 217–223 (1989).

DeWald et al., "Plant regeneration from inbred maize suspensions," VIIth International Congress on Plant Tissue and Cell Culture, p. 12, Abstract No. A1–36 (Jun. 24–29, 1990).

DeWet, J. R. et al., "Cloning of Firefly Luciferase cDNA and the Expression of Active Lucifease in *Escherichia coli," Proc. Nat. Acad. Sci, USA, 82,* 7870–7873 (1985).

Evans, D. A., et al., "Somaclonal Variation—Genetic Basis and Breeding Applications," *Trends Genet., 5,* 46–50 (1989).

Fransz, P., et al., "Cytodifferentiation during callus initiation and somatic embryogenesis in *Zea mays* L.," Ph.D. thesis, U. of Wageningen Press, The Netherlands (1988).

Fromm, M. E., et al., "Stable Transformation of Maize after Gene Transfer by Electroporation," *Nature, 319,* 791–793 (1986).

Fromm, M., et al., "Expression of Genes Transfected into Monocot and Dicot Plant Cells by Electroportion," *Proc. Nat. Acad. Sci. USA, 82,* 5824–5828 (1985).

Gould, O., et al., "Shoot Tip Culture as a Potential Transformation System," Abstracts, Beltwide cotton production research conferences, New Orleans, LA, p. 91 (1988).

Graves, A., et al., "The transformation of *Zea mays* seedlings with *Agrobacterium tumefacians", Plant Mol. Biol., 7,* 43–50 (1986).

Green, C., et al., "Plant Regeneration from Tissue Cultures of Maize," *Crop. Sci., 15,* 417–421 (1975).

Green, C., et al., "Plant Rengeneration in Tissue Cultures of Maize," In: *Maize for Biological Research,* Sheridan, W. F., (ed.) Plant Mol. Biol. Assoc., pp. 367–372 (1982).

Green, C., et al., "Somatic Cell Genetic Systems in Corn," In: *Advances in Gene Technology: Molecular Genetics Plant and Animals,* Academic Press, Inc., pp. 147–157 (1983).

Grimsley, N., et al., "DNA Transfer from Agrobacterium to *Zea mays* or *Brassica* by Agroinfection is Dependent on Bacterial Virulence Functions," *Mol. Gen. Genet., 217,* 309–316 (1989).

Gritz, L., et al., "Plasmid–Encoded Hygromycin B Resistance: The Sequences of Hygromycin B Phosphotransferase Gene and Its Expression in *Escherichia coli* and *Saccharomyces cerevisiae," Gene, 25,* 179–188 (1983).

Guilley, H., et al., "Transcription of Cauliflower Mosaic Virus DNA: Detection of Promotor Sequences, and Characterization of Transcripts," *Cell, 30,* 763–773 (Oct. 1982).

Hooykaas, P. J. J., "Transformation of plant cell via *Agrobacterium," Plant Mol. Biol., 13,* 327–336 (1989).

Horn, M., et al., "Transgenic Plants of Orchard Grass (*Dactylis glomerata L.*) from Protoplasts," *Chem. Abstracts, 110,* p. 208, Abstract No. 89869a (1989).

Jefferson, R., et al., "β–Glucuronidase from *Escherichia coli* as a Gene–Fusion Marker," *Proc. Nat. Acad. Sci. USA 83,* 8447–8451 (1986).

Jefferson, R., et al., "GUS Fusions: β–Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher Plants," *EMBO J. 6,* 3901–3907 (1987).

Jefferson, R., "Assaying chimeric genes in plants: the GUS gene fusion system," *Plant Mol. Biol. Rep., 5,* 387–405 (1987).

Kamo, K., et al., "Establishman and Characterization of Long–Term Embryonic Maize Callus and Cell Suspension Cultures," *Plant Sci., 45,* 111–117 (1986)

Kartha, K., et al., "Transient Expression of Chloramphenicol Acetyl Transferase (CAT) Gene in Barley Cell Cultures and Immature Embryos Through Microprojectile Bombardment," *Plant Cell Rep., 8,* 429–432 (1989).

Klein, T., et al., "Transfer of Foreign Genes into Intact Maize Cells with High–Velocity Microprojectiles," *Proc. Nat. Acad. Sci. USA, 85,* 4305–4309 (1988).

Klein, T. M., et al., "Factors Influencing Gene Delivery into *Zea mays* Cells by High Velocity Microprojectiles," *Bio/Technol., 6,* 559–563 (1988).

Klein, T. M., et al., "High–Velocity Microprojectiles for Delivering Nucleic Acids to Living Cells," *Nature, 327,* 70–73 (1987).

Klein, T., et al., "Genetic Transformation of Maize Cell by Particle Bombardment and the Influence of Methylation on Foreign Gene Expression," in: *Gene Manipulation in Plant Improvement II,* Gustafson, J. P., (ed.), Plenum Press, NY, pp. 265–266 (1990).

Klein, T., et al., "Genetic transformation of Maize Cells by Particle Bombardment," *Plant Physiol., 91,* 440–444 (1989).

Klein, T., et al., "Regulation of Anthocyanin Biosynthetic Genes Introduced into Intact Maize Tissue by Microprojectiles," *Proc. Nat. Acad. Sci. USA, 86,* 6682–6685 (1989).

Kozak, M., "Point Mutations Define a Sequence Flanking the AUG Initiator Codon that Modulates Translation by Eukaryotic Ribosomes," *Cell, 44,* 283–292 (1986).

Lazzeri, P., et al., "In Vitro Genetic Manipulation of Cereals and Grasses," *Ad. Cell Culture 6,* 291–293 (1988).

Lorz, H., et al., "Advances in Tissue Culture and Progress Towards Genetic Transformation of Cereals," *Plant Breeding, 100,* 1–25 (1988).

Lu, C., et al., "Improved Efficiency of Somatic Embryogenesis and Plant Regeneration on Tissue Cultures of Maize (*Zea mays L*)," *Theor. Appl. Genet., 66,* 285–289 (1983).

Ludwig, S., et al., "A Regulatory Gene as a Novel Visible Marker for Maize Transformation," *Science, 247,* 449–450 (1990).

Ludwig, S., et al., "High Frequency Callus Formation from Maize Protoplasts," *Theor. Appl. Genet., 71,* 344–350 (1985).

Ludwig, S., et al., "Lc, a Member of the Maize R Gene Family Responsible for Tissue–Specific Anthocyanin Production, Encodes a Protein Similar to Transcriptional Activators and Contains the myc–Homology Region," *Proc. Nat. Acad. Sci. USA, 86,* 7092–7096 (1989).

Ludwig, S., et al., "Maize R Gene Family: Tissue–Specific Helix–Loop–Helix Proteins," *Cell 62,* 849–851 (1990).

Lutcke, H., et al., "Selection of AUG Initiation Codons Differs in Plants and Animals," *EMBO J., 6,* 43–48 (1987).
McDaniel, C., et al., "Cell–Lineage Patterns in the Shoot Apical Meristem of the Germinating Maize Embryo," *Planta, 175,* 13–22 (1988).
Meadows, M., "Characterization of Cells and Protoplasts of the B73 Maize Cells Line," *Plant Sci. Lett., 28,* 337–348 (1982/83).
Mendel, R., et al., "Delivery of Foreign Genes to Intact Barley Cell by High–Velocity Microprojectiles," *Theor. Appl. Genet., 78,* 31–34 (1989).
Murakami, T., et al., "The Bialaphos Biosynthetic Genes of *Streptomyces hygrosopicus:* Molecular Cloning and Characterization of the Gene Cluster," *Mol. Gen. Genet., 205,* 42–50 (1986).
Nelson, T., "New Horses for Monocot Gene Jockeys," *The Plant Cell, 2,* 589 (1990).
Neuffer, "Growing Maize for Genetic Purposes," Maize for Biological Research, Plant Mol. Biol. Assoc., pp. 19–30 (1988).
Odell, J., et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter," *Nature, 313,* 810–811 (1985).
Ohta, Y., "High–Efficiency Genetic Transformation of Maize by a Mixture of Pollen and Exogenous DNA," *Proc. Nat. Acad. Sci. USA, 83,* 715–719 (1986).
Ozias–Akins, P., et al., "In vitro regeneration and genetic manipulation of grasses," *Physiol. Plant, 73, 565–569 (1988).*
Ozias–Akins, P., et al., "Progress and Limitations in the Culture of Cereal Protoplasts," *Trends in Biotechnol., 2,* 119–123 (1984).
Phillips, R. L., et al., "Cell/Tissue Culture and In Vitro Manipulation," In: *Corn and Corn Improvement,* 3rd edition, Sprague, G. F., et al., (eds.), Agronomy Soc. Amer., pp. 345–387 (1988).
Poehlman, J. "Breeding Corn (Maize)," In: *Breeding Field Crops,* 3rd edition, AVI Publishing Co., Westport CN, pp. 469–471, 477–481 (1986).
Potrykus, I., "Gene Transfer to Cereals: An Assessment," Bio/Technol., 8, 535–542 (Jun. 1990).
Potrykus, I., "Gene Transfer to Cereals: an Assessment," *Trends Biotechnol., 7,* 269–273 (Oct. 1989).
Potrykus, I., et al., "Callus formation from stem protoplasts of corn (*Zea mays L.*)" *Mol. Gen. Genet., 156,* 347–350 (1977).
Prioli, L. M., et al., "Plant Regeneration and Recovery of Fertile Plants from Protoplasts of Maize (*Zea mays L.*)," *Bio./Technol, 7,* 589–594 (Jun. 1989).
Rhodes, C. A., et al., "Genetically Transformed Maize Plants from Protoplasts," *Science, 240,* 204–207 (Apr. 8, 1988).
Rhodes, C. A., et al., "Plant Regeneration from Protoplasts Isolated from Embryogenic Maize Cell Cultures," *Bio./Technol., 6,* 56–60 (Jan. 1988).
Rhodes, C. A., "Corn: From Protoplasts to Fertle Plants," *Bio./Technol., 7,* 548 (Jun. 1989).
Sanford, J. C., et al., "Biolistic Plant Transformation," *Physiol. Plant., 79,* 206–209 (1990).
Sanford, J. C., et al., "Attempted Pollen–Mediated Plant Transformation Employing Genomic Donor DNA," *Theor. appl. Genet., 69,* 571–574 (1985).
Sanford, J. C., et al., "Delivery of Substances into Cells and Tissues using a Particle Bombardment Process," *Paticulate Sci. Technol., 5, 27–37 (1987).*
Schmidt, A., et al., "Media and environmental effects of phenolics production from tobacco cell cultures," *Chem. Abstracts, 110,* p. 514, Abstract No. 230156z (1989).
Shillito, R. D., et al., "Regeneration of Fertile Plants From Protoplasts of Elite Inbred Maize," *Bio/Technol. 7,* 581–587 (Jun. 1989).
Smith, R., et al., "Shoot apex explant for transformation," *Plant Physiol., 86,* p. 108, Abstract No. 646 (1988).
Spencer et al., "Bialaphos Selection of Stable Transformations from Maize Cell Culture," *Theor. Appl. Genet., 79,* 625–631 (May 1990).
Spencer, T. M., et al., "Selection of Stable Transformants from Maize Suspension Cultures using the Herbicide Bialophos," Poster presentation, FASEB Plant Gene Expression Conference, Copper Mountain, Colorado (Aug. 8, 1989).
Thompson, C., et al., "Characterization of the Herbicide–Resistance Gene *bar* from *Streptomyces hygroscopicus,*" *EMBO J., 6,* 2519–2523 (1987).
Tomes, D. T., et al., "Transgenic Tobacco Plants and their Progeny Derived by Microprojectile Bombardment of Tobacco Leaves," *Plant Mol. Biol., 14,* 261–268 (Feb. 1990).
Twell, D., et al., "Transient Expression of Chimeric Genes Delivered into Pollen by Microprojectile Bombardment of *Plant Physiol., 91,* 1271–1274 1989).
Ulian, E., et al., "Transformation of Plants via the Shoot Apex," *In Vitro Cell. Dev. Biol., 9,* 951–954 (1988).
Vasil, V., et al., "Plant Regeneration from Friable Embryonic Callus and Cell Suspension Cultures of *Zea mays L.," J. Plant Physiol., 124,* 399–408 (1986).
Walbot, V., et al., "Molecular genetics of corn," In: *Corn and Corn Improvement,* 3rd edition, Sprauge, G. F. et al., (eds.), American Soc. Agronomy, Madison, WI, pp. 389–430 (1988).
Wang, Y., et al., "Transient Expression of Foreign Genes in Rice, Wheat and Soybean Cells Following Particle Bombardment," *Plant Mol. Biol., 11,* 433–439 (1988).
Weising, K., et al., "Foreign Genes in Plants: Transfer, Structure, Expression and Applications," *Ann. Rev. Genet., 22,* 421–478 (1988).
White, J., et al., "A Cassette Containing the bar Gene for *Streptomyces hygroscopicus:* a Selectable Marker for Plant Transformation," *Nuc. Acid. Res., 18,* 1062 (1989).
Armstrong et. al. *Plants* "Establishment & maintenance of friable, embryogenic maize callus & the involvement of L–proline." vol. 164 pp. 207–214 (1985).
Klein et al. *Biotechnology* "Factors influencing Gene Delivery into *Zea Mays* cells by High–velocity Microprojectiles" vol. 6 pp. 559–563 (1988).

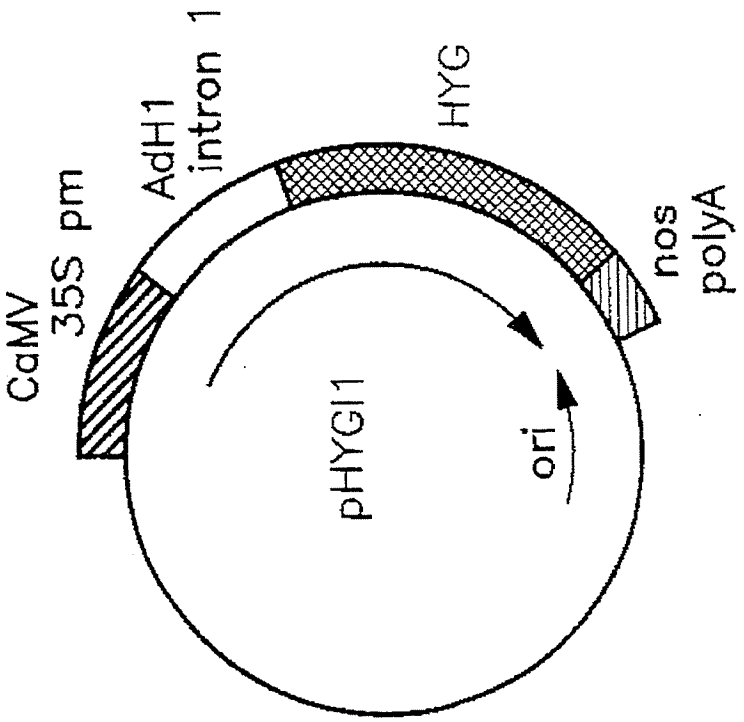

CaMV
35S pm
AdH1
intron 1
HYG
nos
polyA
pHYGI1
ori

FIG. 1A

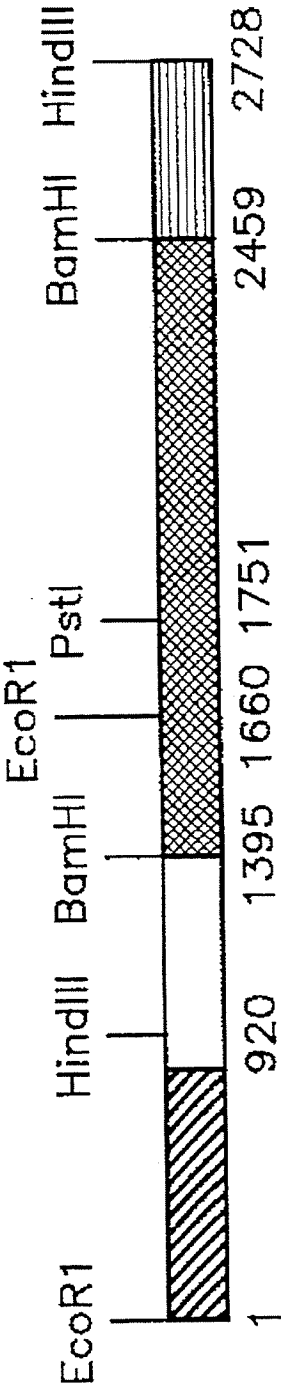
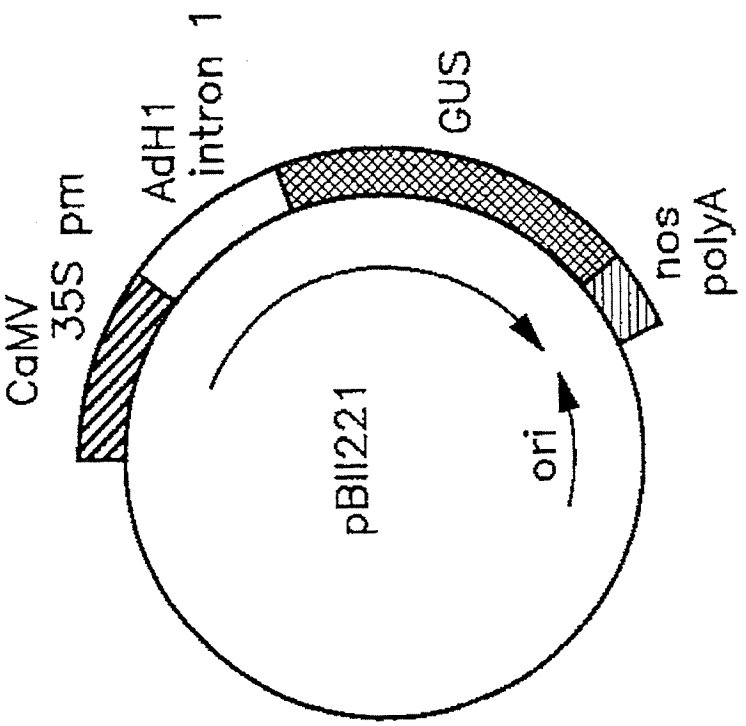

HindIII
BamHI
2728
2459
EcoR1
PstI
1751
1660
BamHI
1395
HindIII
920
EcoR1
1

FIG. 1B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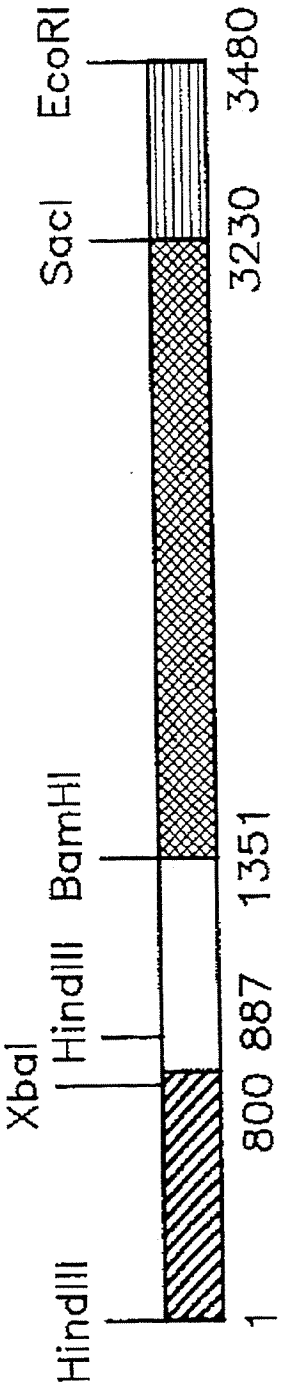
AdH1
intron 1
GUS
nos
polyA
pBI1221
ori

FIG. 2A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SacI
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BamHI
1351
XbaI
HindIII
887
800
HindIII
1

FIG. 2B

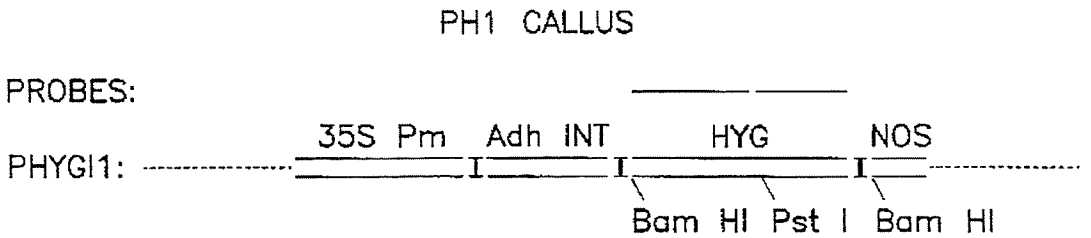

PH1 CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 3A

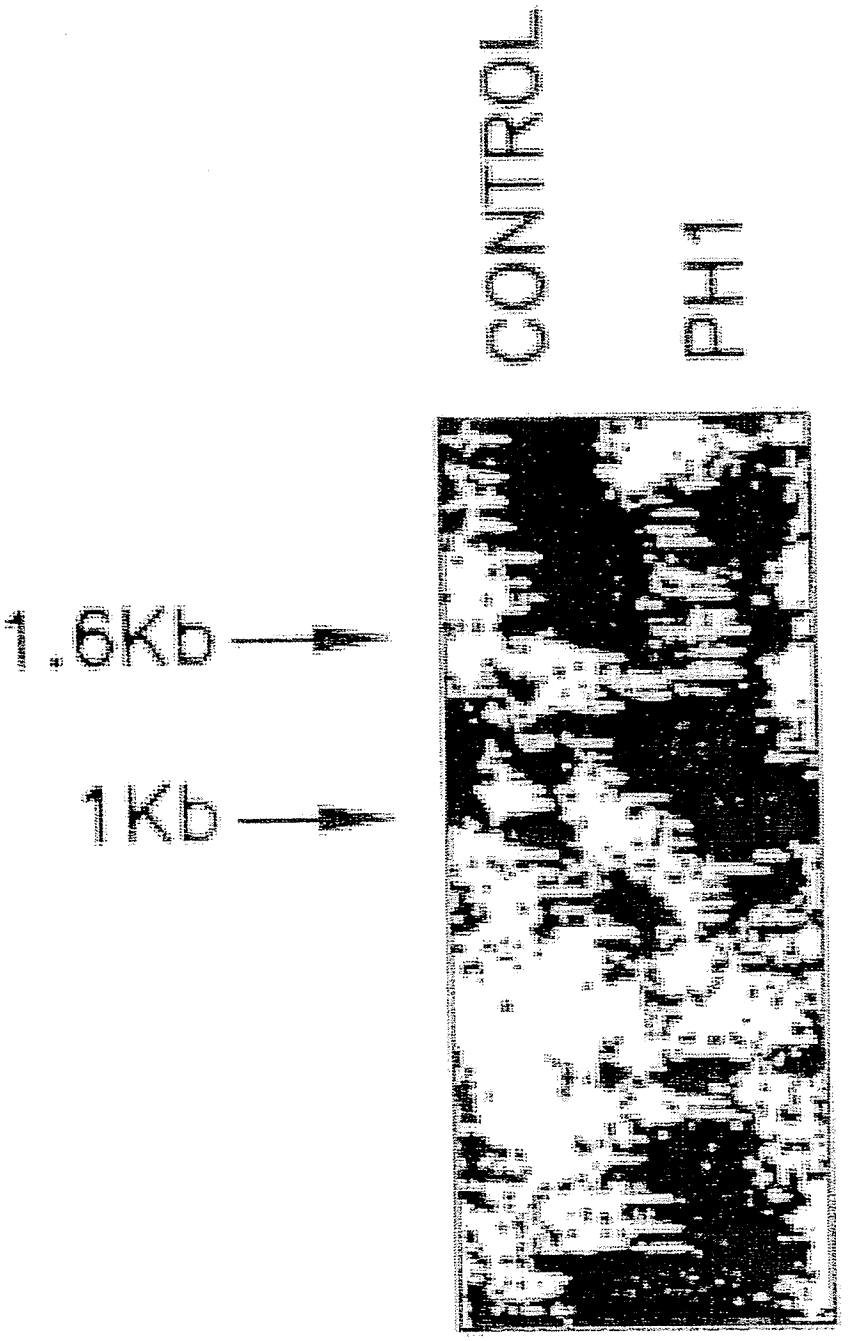


FIG. 3B

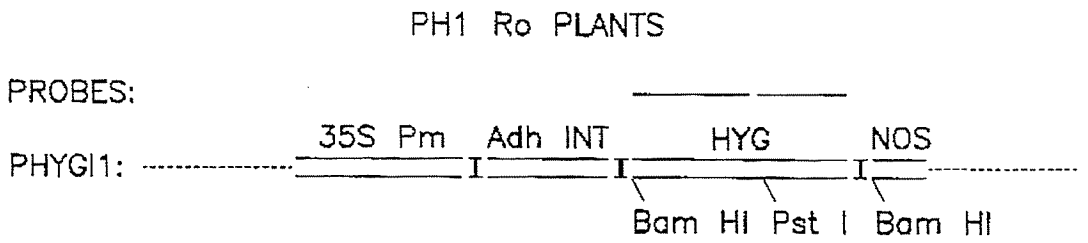

PH1 Ro PLANTS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 4A

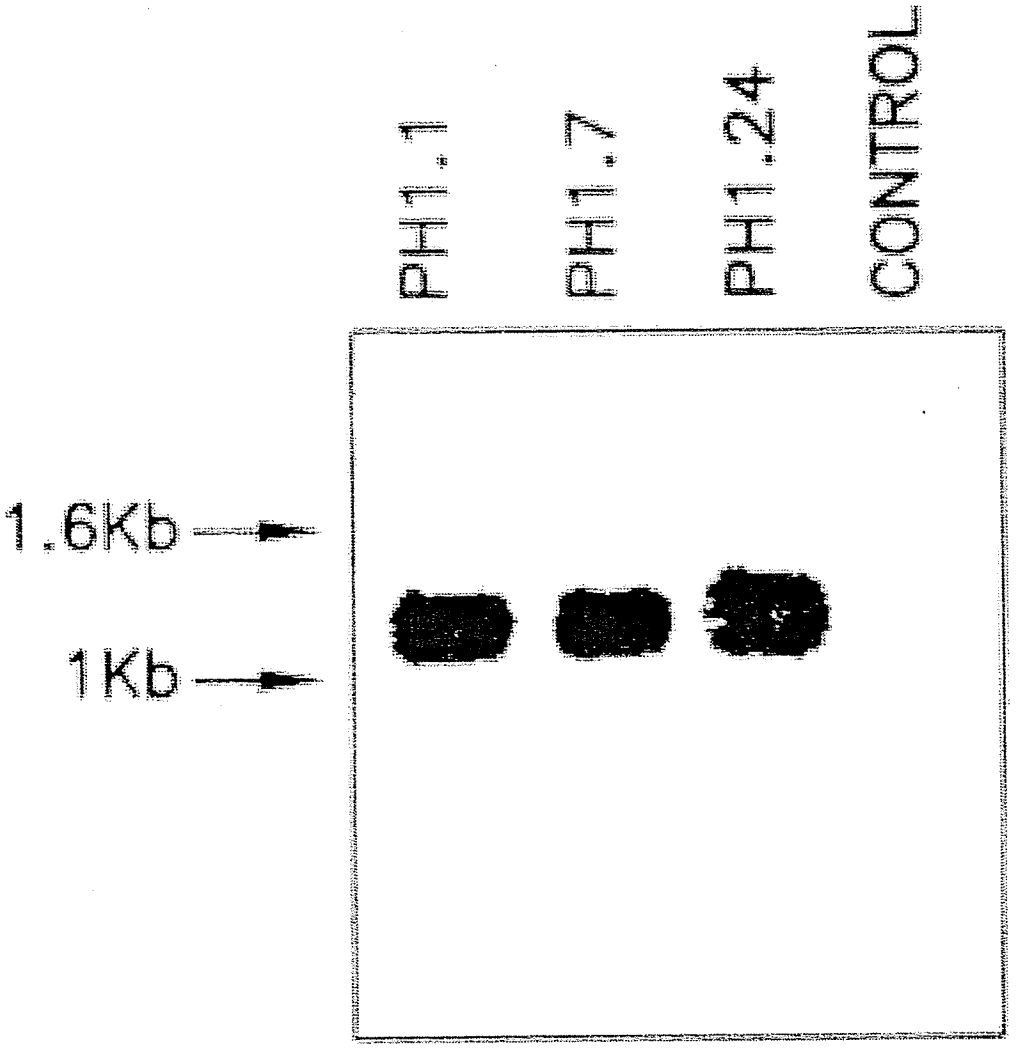


FIG. 4B

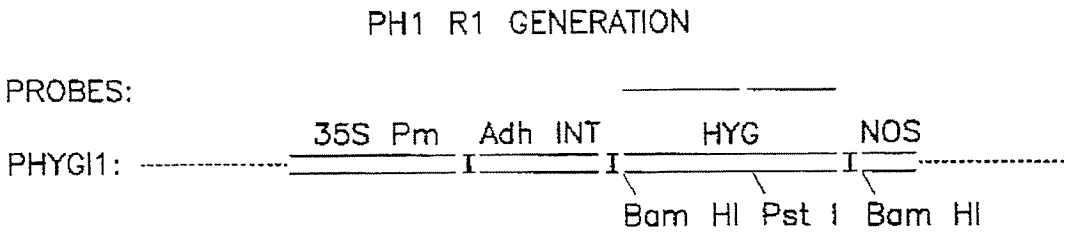

PH1 R1 GENERATION
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 5A

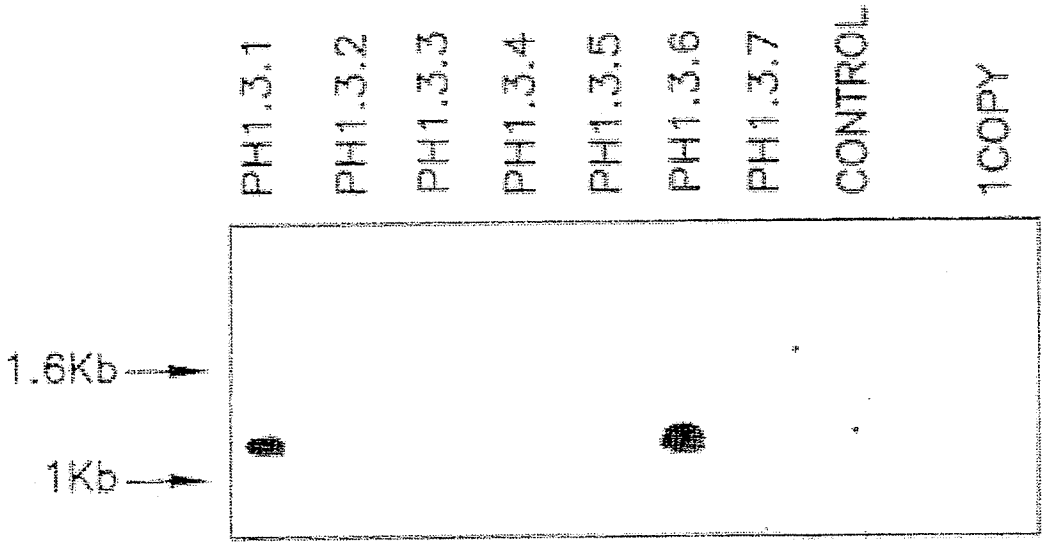
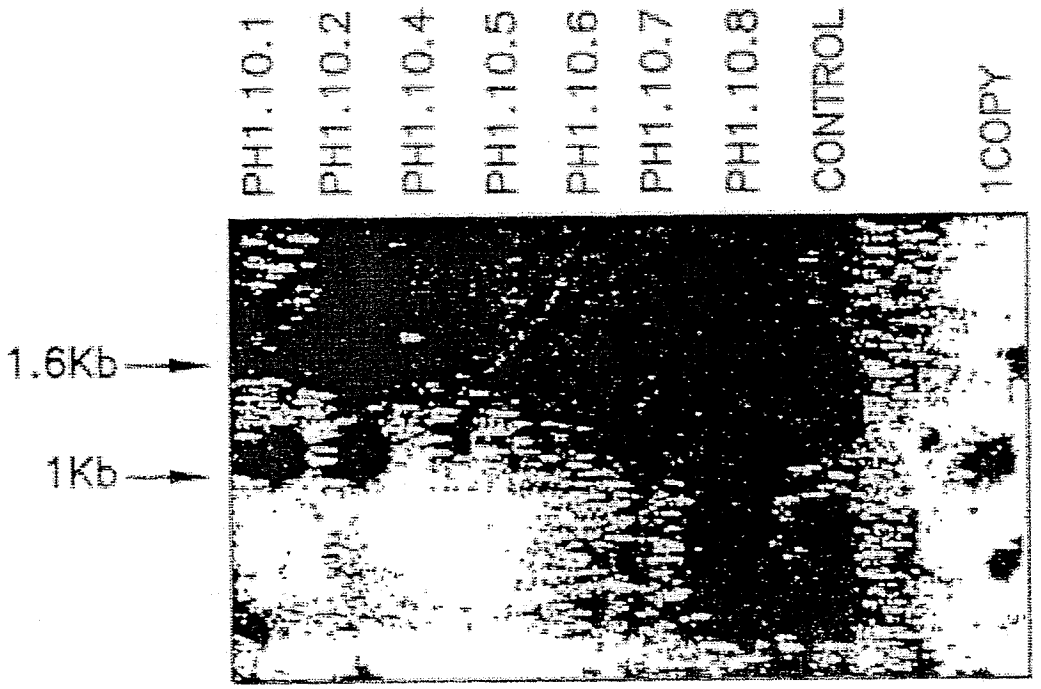

PH1.3.1
PH1.3.2
PH1.3.3
PH1.3.4
PH1.3.5
PH1.3.6
PH1.3.7
CONTROL
1COPY
1.6Kb
1Kb

FIG. 5B


PH1.10.1
PH1.10.2
PH1.10.4
PH1.10.5
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PH1.10.7
PH1.10.8
CONTROL
1COPY
1.6Kb
1Kb

FIG. 5C

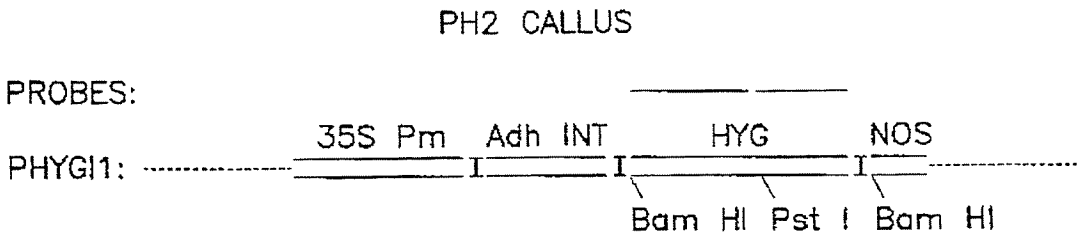
PH2 CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 6A

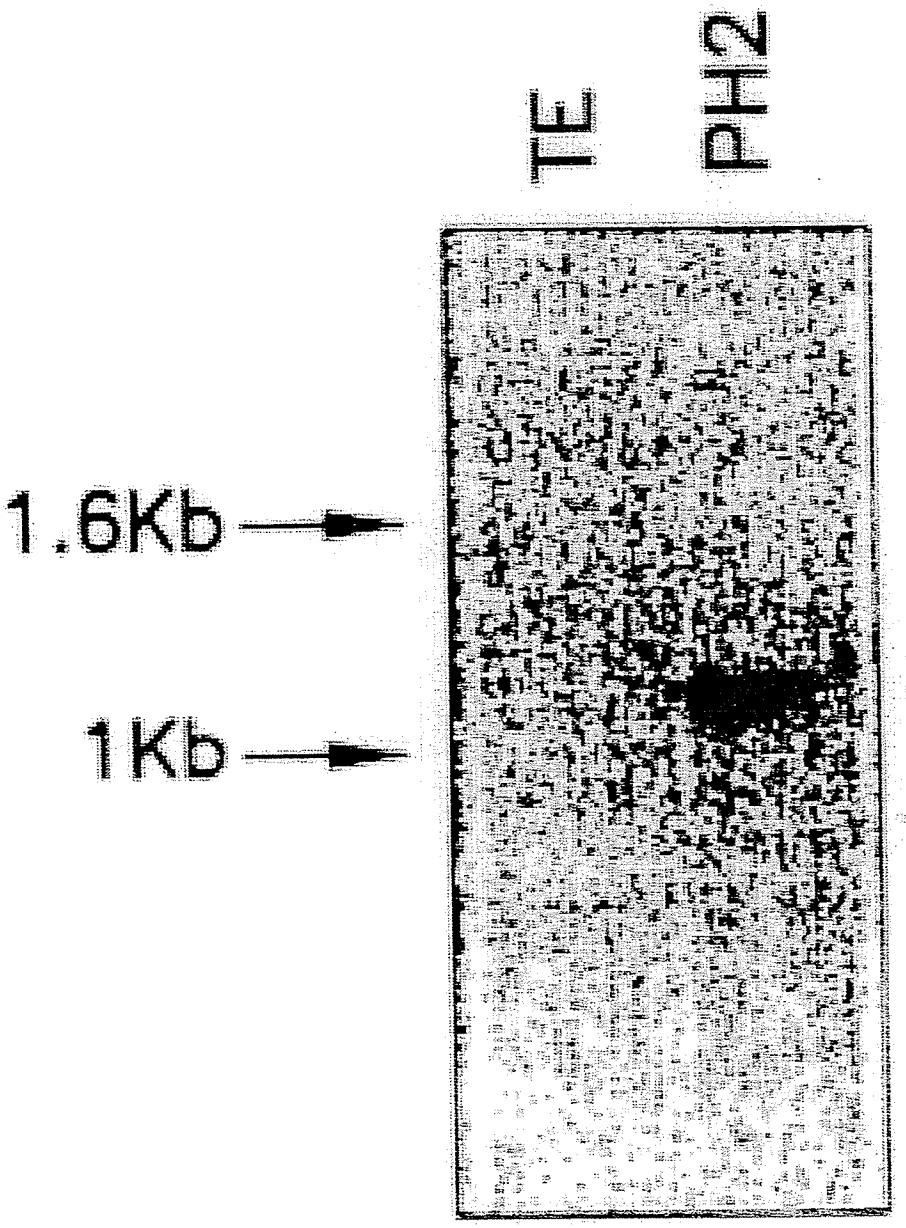

FIG. 6B

METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS

This is a division of application Ser. No. 07/974,379, filed Nov. 10, 1992, which is a continuation of application Ser. No. 07/467,983, filed Jan. 22, 1990, abandoned.

BACKGROUND OF THE INVENTION

This invention relates to fertile transgenic plants of the species *Zea mays* (oftentimes referred to herein as maize or corn). The invention further relates to producing transgenic plants via particle bombardment and subsequent selection techniques which have been found to produce fertile transgenic plants.

Genetic engineering of plants, which entails the isolation and manipulation of genetic material (usually in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells, offers considerable promise to modern agriculture and plant breeding. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals, chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques. Once a gene has been identified, cloned, and engineered, it is still necessary to introduce it into a plant of interest in such a manner that the resulting plant is both fertile and capable of passing the gene on to its progeny.

A variety of methods have been developed and are currently available for the transformation of various plants and plant cells with DNA. Generally these plants have been dicotyledonous, and some success has been reported with certain of the monocotyledonous cereals. However, some species have heretofore proven untransformable by any method. Thus, previous to this discovery, no technology had been developed which would permit the production of stably transformed *Zea mays* plants in which the transforming DNA is heritable thereof. This failure in the art is well documented in the literature and has been discussed in a number of recent reviews (Potrykus, 1989; Weising et al., 1988; Cocking et al., 1987).

European Patent Publns. 270,356 (McCabe et al.) and 275,069 (Arntzen et al.) describe the introduction of DNA into maize pollen followed by pollination of maize ears and formation of seeds. The plants germinated from these seeds are alleged to contain the introduced DNA, but there is no suggestion that the introduced DNA was heritable, as has been accomplished in the present invention. Only if the DNA introduced into the corn is heritable can the corn be used in breeding programs as required for successful commercialization of transgenic corn.

Graves et al. (1986) claims *Agrobacterium*-mediated transformation of *Zea mays* seedlings. The alleged evidence was based upon assays known to produce incorrect results.

Despite extensive efforts to produce fertile transformed corn plants which transmit the transforming DNA to progeny, there have been no reported successes. Many previous failures have been based upon gene transfer to maize protoplasts, oftentimes derived from callus, liquid suspension culture cells, or other maize cells using a variety of transformation techniques. Although several of the techniques have resulted in successful transformation of corn cells, the resulting cells either could not be regenerated into corn plants or the corn plants produced were sterile (Rhodes et al. 1988). Thus, while maize protoplasts and some other cells have previously been transformed, the resulting transformants could not be regenerated into fertile transgenic plants.

On the other hand, it has been known that at least certain corn callus can be regenerated to form mature plants in a rather straightforward fashion and that the resulting plants were often fertile. However, no stable transformation of maize callus was ever achieved, i.e. there were no techniques developed which would permit a successful stable transformation of a regenerable callus. An example of a maize callus transformation technique which has been tried is the use of Agrobacterium mediated transfer.

The art was thus faced with a dilemma. While it was known that corn protoplast and suspension culture cells could be transformed, no techniques were available which would regenerate the transformed protoplast into a fertile plant. While it was known that corn callus could be regenerated into a fertile plant, there were no techniques known which could transform the callus, particularly while not destroying the ability of the callus both to regenerate and to form fertile plants.

Recently, a new transformation technique has been created based upon the bombardment of intact cells and tissues with DNA-coated microprojectiles. The technique, disclosed in Sanford et al. (1987) as well as in EPO Patent Publication 331,855 of J. C. Sanford et al. based upon U.S. Ser. No. 161,807, filed Feb. 29, 1988, has been shown effective at producing transient gene expression in some plant cells and tissues including those from onion, maize (Klein et al. 1988a), tobacco, rice, wheat, and soybean, and stable expression has been obtained in tobacco and soybeans. In fact, stable expression has been obtained by bombardment of suspension cultures of *Zea mays* Black Mexican Sweet (Klein et al. 1989) which cultures are, however, non-regenerable suspension culture cells, not the callus culture cells used in the process of the present invention.

No protocols have been published describing the introduction of DNA by a bombardment technique into cultures of regenerable maize cells of any type. No stable expression of a gene has been reported by means of bombardment of corn callus followed by regeneration of fertile plants and no regenerable fertile corn has resulted from DNA-coated microprojectile bombardment of the suspension cultures. Thus, the art has failed to produce fertile transformed corn plants heretofore.

A further stumbling block to the successful production of fertile transgenic maize plants has been in selecting those few transformants in such a manner that neither the regeneration capacity nor the fertility of the regenerated transformant are destroyed. Due to the generally low level of transformants produced by a transformation technique, the need for selection of the transformants is self-evident. However, selection generally entails the use of some toxic agent, e.g. herbicide or antibiotic, which can effect either the regenerability or the resultant plant fertility.

It is thus an object of the present invention to produce fertile, stably transgenic, *Zea mays* plants and seeds which transmit the introduced gene to progeny. It is a further object to produce such stably transgenic plants and seeds by a particle bombardment and selection process which results in a high level of viability for a few transformed cells. It is a further object to produce fertile stably transgenic plants of other graminaceous cereals besides maize.

REFERENCES CITED

Armstrong, C L, et al. (1985) J Planta 164:207–214

Callis, J, et al. (1987) Genes & Develop 1:1183–1200

M. Bouan et al., (1983) Nuc Acids Res., 11:369(1983)

Chu, C C, et al. (1975) Sci Sin (Peking) 18:659–668

Cocking, F, et al. (1987) Science 236:1259–1262

DeWet et al. (1985) Proc Natl Sci USA 82:7870–7873

Freeling, J C, et al. (1976) Maydica XXI:97–112

Graves, A, et al. (1986) Plant Mol Biol 7:43–50

Green, C, et al. (1975) Crop Sci 15:417–421

Green, C E, (1982) Plant Tissue Culture, A Fujiwara ed. Maruzen, Tokyo, Japan pp 107–8

Green, C, et al. (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 367–372

Gritz, L, et al. (1983) Gene 25:179–188

Guilley, H, et al. (1982) Cell 30:763–773

Jefferson, R, et al. (1987) EMBO J 6:3901–3907)

Kamo, K, et al. (1985) Bot Gaz 146:327–334

Klein, T, et al. (1989) Plant Physiol 91:440–444

Klein, T, et al. (1988a) Proc Natl Acad Sci USA 85:4305–9

Klein, T, et al. (1988b) Bio/Technology 6:559–563

Lu, C, et al. (1982) Theor Appl Genet 62:109–112

McCabe, D, et al. (1988) Bio/Technology 6:923–926

Murashige, T, et al. (1962) Physiol Plant 15:473–497

Neuffer, M, (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 19–30

Phillips, R, et al. (1988) Corn and Corn Improvement, 3rd ed., Agronomy Soc Amer, pp 345–387

Potrykus, I (1989) Trends in Biotechnology 7:269–273

Rhodes, C A, et al. (1988) Science 240:204–7

Sambrook, J, et al (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press

Sanford, J, et al. (1987) J Part Sci & Techn 5:27–37

Weising, K,.et al., (1988) Ann Rev of Genetics 22:421–478

Yanisch-Perron, L, et al. (1985) Gene 33:109–119

SUMMARY OF THE INVENTION

The present invention relates to fertile transgenic *Zea mays* plants containing heterologous DNA, preferably chromosomally integrated heterologous DNA, which is heritable by progeny thereof.

The invention further relates to all products derived from transgenic *Zea mays* plants, plant cells, plant parts, and seeds.

The invention further relates to transgenic *Zea mays* seeds stably containing heterologous DNA and progeny which inherit the heterologous DNA.

The invention further relates to a process for producing fertile transgenic *Zea mays* plants containing heterologous DNA. The process is based upon microprojectile bombardment, selection, and plant regeneration techniques.

The invention further relates to a process for producing fertile transformed plants of graminaceous plants other than *Zea mays* which have not been reliably transformed by traditional methods such as electroporation, *Agrobacterium*, injection, and previous ballistic techniques.

The invention further relates to regenerated fertile mature maize plants from transformed embryogenic tissue, transgenic seeds produced therefrom, and R1 and subsequent generations.

In preferred embodiments, this invention produces the fertile transgenic plants by means of a DNA-coated microprojectile bombardment of clumps of friable embryogenic callus, followed by a controlled regimen for selection of the transformed callus lines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**A shows a map of plasmid vector pHYGI1 utilized in Example I. FIG. **1**B shows the relevant part of pHYGI1 encompassing the HPT coding sequence and associated regulatory elements. The base pair numbers start from the 5' nucleotide in the recognition sequence for the indicated restriction enzymes, beginning with the EcoRI site at the 5' end of the CaMV 35S promoter.

FIG. **2**A shows a map of plasmid vector pBII221 utilized in Example I.

FIG. **2**B shows the relevant part of pBII221 encompassing the GUS coding sequence and associated regulatory elements.

FIG. **3**A depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PH1 callus.

FIG. **3**B is a Southern blot of DNA isolated from the PH1 callus line and an untransformed control callus line.

FIG. **4**A depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PH1 RO plants.

FIG. **4**B is a Southern blot of leaf DNA isolated from Ro plants regenerated from PH1 and untransformed callus.

FIG. **5**A depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PHI R1 plants.

FIG. **5**B and **5**C are Southern blots of leaf DNA isolated from R1 progeny of PH1 Ro plants and untransformed Ro plants.

FIG. **6**A depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PH2 callus.

FIG. **6**B is a Southern blot of DNA isolated from the PH2 callus line and an untransformed control callus line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the production of fertile transgenic plants and seeds of the species *Zea mays* and to the plants, plant tissues, and seeds derived from such transgenic plants, as well as the subsequent progeny and products derived therefrom. The transgenic plants produced herein include all plants of this species, including field corn, popcorn, sweet corn, flint corn and dent corn.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant which contains heterologous DNA that was introduced into plant material by a process of genetic engineering, or which was initially introduced into a plant species by such a process and was subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions.

By "heritable" is meant that the DNA is capable of transmission through a complete sexual cycle of a plant, i.e. passed from one plant through its gametes to its progeny plants in the same manner as occurs in normal corn.

The transgenic plants of this invention may be produced by (i) establishing friable embryogenic callus from the plant to be transformed, (ii) transforming said cell line by a microprojectile bombardment technique, (iii) controllably identifying or selecting transformed cells, and (iv) regenerating fertile transgenic plants from the transformed cells. Some of the plants of this invention may be produced from the transgenic seed produced from the fertile transgenic plants using conventional crossbreeding techniques to develop commercial hybrid seed containing heterologous DNA.

I. Plant Lines and Tissue Cultures

The cells which have been found useful to produce the fertile transgenic maize plants herein are those callus cells which are regenerable, both before and after undergoing a selection regimen as detailed further below. Generally, these cells will be derived from meristematic tissue which contain cells which have not yet terminally differentiated. Such tissue in graminaceous cereals in general and in maize, in particular, comprise tissues found in juvenile leaf basal regions, immature tassels, immature embryos, and coleoptilar nodes. Preferably, immature embryos are used. Methods of preparing and maintaining callus from such tissue and plant types are well known in the art and details on so doing are available in the literature, c.f. Phillips et al. (1988), the disclosure of which is hereby incorporated by reference.

The specific callus used must be able to regenerate into a fertile plant. The specific regeneration capacity of particular callus is important to the success of the bombardment/selection process used herein because during and following selection, regeneration capacity may decrease significantly. It is therefore important to start with cultures that have as high a degree of regeneration capacity as possible. Callus which is more than about 3 months and up to about 36 months of age has been found to have a sufficiently high level of regenerability and thus is currently preferred. The regenerative capacity of a particular culture may be readily determined by transferring samples thereof to regeneration medium and monitoring the formation of shoots, roots, and plantlets. The relative number of plantlets arising per Petri dish or per gram fresh weight of tissue may be used as a rough quantitative estimate of regeneration capacity. Generally, a culture which will produce at least one plant per gram of callus tissue will be preferred.

While maize callus cultures can be initiated from a number of different plant tissues, the cultures useful herein are preferably derived from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1–3 mm in length. This length generally occurs about 9–14 days after pollination. Under aseptic conditions, the embryos are placed on conventional solid media with the embryo axis down (scutellum up). Callus tissue appears from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" is meant that the tissue is easily dispersed without causing injury to the cells. Tissue with this morphology is then transferred to fresh media and subcultured on a routine basis about every two weeks.

The callus initiation media is solid because callus cannot be readily initiated in liquid medium. The initiation/maintainence media is typically based on the N6 salts of Chu et al. (1975) as described in Armstrong et al. (1985) or the MS salts of Murashige et al. (1962). The basal medium is supplemented with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth. The cultures are generally maintained in the dark, though low light levels may also be used. The level of synthetic hormone 2,4-D, necessary for maintainence and propagation, should be generally about 0.3 to 3.0 mg/l.

Although successful transformation and regeneration has been accomplished herein with friable embryogenic callus, this is not meant to imply that other transformable regenerable cells, tissue, or organs cannot be employed to produce the fertile transgenic plants of this invention. The only actual requirement for the cells which are transformed is that after transformation they must be capable of regeneration of a plant containing the heterologous DNA following the particular selection or screening procedure actually used.

II. DNA Used for Transformation

The heterologous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of a plasmid and contains coding regions of beneficial heterologous DNA with flanking regulatory sequences which serve to promote the expression of the heterologous DNA present in the resultant corn plant. "Heterologous DNA" is used herein to include all synthetically engineered or biologically derived DNA which is introduced into a plant by man by genetic engineering, including but not limited to, nonplant genes, modified genes, synthetic genes, portions of genes, as well as DNA and genes from maize and other plant species.

The compositions of and methods for constructing heterologous DNA for successful transformations of plants is well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the heterologous DNA useful herein. The specific composition of the DNA is not central to the present invention and the invention is not dependent upon the composition of the specific transforming DNA used. Weising et al. (1988), the subject matter of which is incorporated herein by reference, describes suitable DNA components thereof which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, as well as provides suitable references for compositions thereof. Sambrook et al. (1989) provides suitable methods of construction.

Generally the heterologous DNA will be relatively small, i.e. less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the DNA increases.

Suitable heterologous DNA for use herein includes all DNA which will provide for, or enhance, a beneficial feature of the resultant transgenic corn plant. For example, the DNA may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. For example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; bacterial ESPS synthase for resistance to glyphosate herbicide; chitinase or glucan endo-1,3-B-glucosidase for fungicidal properties. Also, the DNA may be introduced to act as a genetic tool to generate mutants and/or assist in the identification, genetic tagging, or isolation of segments of corn DNA. Additional examples may be found in Weising, supra.

The heterologous DNA to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a cotransformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Weising et al, supra. A preferred selectable marker gene is the hygromycin B phosphotransferase (HPT) coding sequence, which may be derived from *E. coli*. Other selectable markers known in the art include aminoglycoside phosphotransferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin, and G418, as well as those genes which code for resistance or tolerance to glyphosate, methotrexate, imidazolinones, sulfonylureas, bromoxynil, dalapon, and the like. Those selectable marker genes which confer herbicide resistance or tolerance are also of commercial utility in the resulting transformed plants.

Reporter genes which encode for easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g. phenotypic change or enzymatic activity. Examples of such genes are provided in Weising et al, supra. Preferred genes include the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the beta-glucuronidase gene of the uidA locus of *E. coli*, and the luciferase genes from firefly *Photinus pyralis*.

The regulatory sequences useful herein include any constitutive, inducible, tissue or organ specific, or developmental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al, supra. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *Agrobacterium tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase promoter from corn; light inducible promoters such as, ribulose-biphosphate-carboxylase small subunit gene from a variety of species; and the major chlorophyll a/b binding protein gene promoter; 35S and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present on the DNA. Such elements may or may not be necessary for the function of the DNA, although they can provide a better expression or functioning of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the plant. For example, the maize AdhlS first intron may be placed between the promoter and the coding sequence of a particular heterologous DNA. This intron, when included in a DNA construction, is known to generally increase expression in maize cells of a protein. (Callis et al. 1987) However, sufficient expression for a selectable marker to perform satisfactorily can often be obtained without an intron. (Klein et al. 1989) An example of an alternative suitable intron is the shrunken-1 first intron of Zea mays. These other elements must be compatible with the remainder of the DNA constructions.

To determine whether a particular combination of DNA and recipient plant cells are suitable for use herein, the DNA may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the DNA has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (GUS) gene (Jefferson et al. 1987). In the case of the microprojectile bombardment transformation process of the present invention, a suitable time for conducting the assay is about 2–3 days after bombardment. The use of transient assays is particularly important when using DNA components which have not previously been demonstrated or confirmed as compatible with the desired recipient cells.

III. DNA Delivery Process

The DNA can be introduced into the regenerable maize callus cultures via a particle bombardment process. A general description of a suitable particle bombardment instrument is provided in Sanford et al. (1987), the disclosure of which is incorporated herein by reference. While protocols for the use of the instrument in the bombardment of maize non-regenerable suspension culture cells are described in Klein et al. (1988a, 1988b, and 1989), no protocols have been published for the bombardment of callus cultures or regenerable maize cells.

In a microprojectile bombardment process, also referred to as a biolistic process, the transport of the DNA into the callus is mediated by very small particles of a biologically inert material. When the inert particles are coated with DNA and accelerated to a suitable velocity, one or more of the particles is able to enter into one or more of the cells where the DNA is released from the particle and expressed within the cell. While some of the cells are fatally damaged by the bombardment process, some of the recipient cells do survive, stably retain the introduced DNA, and express it.

The particles, called microprojectiles, are generally of a high density material such as tungsten or gold. They are coated with the DNA of interest. The microprojectiles are then placed onto the surface of a macroprojectile which serves to transfer the motive force from a suitable energy source to the microprojectiles. After the macroprojectile and the microprojectiles are accelerated to the proper velocity, they contact a blocking device which prevents the macroprojectile from continuing its forward path but allows the DNA-coated microprojectiles to continue on and impact the recipient callus cells. Suitable such instruments may use a variety of motive forces such as gunpowder or shock waves from an electric arc discharge J. C. Sanford et al., *J. Particle Science and Technology*, 5,27 (1987). An instrument in which gunpowder is the motive force is currently preferred and such is described and further explained in Sanford et al. (1987), the disclosure of which is incorporated herein by reference.

A protocol for the use of the gunpowder instrument is provided in Klein et al. (1988a, b) and involves two major steps. First, tungsten microprojectiles are mixed with the DNA, calcium chloride, and spermidine free base in a specified order in an aqueous solution. The concentrations of the various components may be varied as taught. The currently preferred procedure entails exactly the procedure of Klein et al. (1988b) except for doubling the stated

optimum DNA concentration. Secondly, in the actual bombardment, the distance of the recipient cells from the end of the barrel as well as the vacuum in the sample chamber. The currently preferred procedure for bombarding the callus entails exactly the procedure of Klein et al. (1988b) with the recipient tissue positioned 5 cm below the stopping plate tray.

The callus cultures useful herein for generation of transgenic plants should generally be about 3 months to 3 years old, preferably about 3 to 18 months old. Callus used for bombardment should generally be about midway between transfer periods and thus past any "lag" phase that might be associated with a transfer to a new media, but also before reaching any "stationary" phase associated with a long time on the same plate.

The specific tissue subjected to the bombardment process is preferably taken about 7–10 days after subculture, though this is not believed critical. The tissue should generally be used in the form of pieces of about 30 to 80, preferably about 40 to 60, mg. The clumps are placed on a petri dish or other surface and arranged in essentially any manner, recognizing that (i) the space in the center of the dish will receive the heaviest concentration of metal-DNA particles and the tissue located there is likely to suffer damage during bombardment and (ii) the number of particles reaching a cell will decrease (probably exponentially) with increasing distance of the cell from the center of the blast so that cells far from the center of the dish are not likely to be bombarded and transformed. A mesh screen, preferably of metal, may be laid on the dish to prevent splashing or ejection of the tissue. The tissue may be bombarded one or more times with the DNA-coated metal particles.

IV. Selection Process

Once the calli have been bombarded with the DNA and the DNA has penetrated some of the cells, it is necessary to identify and select those cells which both contain the heterologous DNA and still retain sufficient regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively and preferably, when a selectable marker gene has been transmitted along with or as part of the heterologous DNA, those cells of the callus which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells while simultaneously inhibiting the growth of the non-transformed cells. The situation is complicated by the fact that the vitality of individual cells in a population is often highly dependent on the vitality of neighboring cells. Also, the selection conditions must not be so severe that the plant regeneration capacity of the callus cells and the fertility of the resulting plant are precluded. Thus the effects of the selection agent on cell viability and morphology should be evaluated. This may be accomplished by experimentally producing a growth inhibition curve for the given selective agent and tissue being transformed beforehand. This will establish the concentration range which will inhibit growth.

When a selectable marker gene has been used, the callus clumps may be either allowed to recover from the bombardment on non-selective media or, preferably, directly transferred to media containing that agent.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably the concentration of the agent is initially such that about a 5–40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells to preferentially grow and divide while inhibiting untransformed cells, but not to the extent that growth of the transformed cells is prevented. Once the few individual transformed cells have grown sufficiently the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of nontransformed cells habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative maize transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells. The callus may also be cultured on non-selective media at various times during the overall selection procedure.

Once a callus sector is identified as a putative transformant, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e. Southern blot or polymerase chain reaction (PCR) or the like.

V. Regeneration of Plants and Production of Seed

Cell lines which have been shown to be transformed must then be regenerated into plants and the fertility of the resultant plants determined. Transformed lines which test positive by genotypic and/or phenotypic analysis are then placed on a media which promotes tissue differentiation and plant regeneration. Regeneration may be carried out in accordance with standard procedures well known in the art. The procedures commonly entail reducing the level of auxin which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. One example of such a regeneration procedure is described in Green et al. (1981). The plants are grown to maturity in a growth room or greenhouse and appropriate sexual crosses and selfs are made as described by Neuffer (1981).

Regeneration, while important to the present invention, may be performed in any conventional manner. If a selectable marker has been transformed into the cells, the selection agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are transformed. Since regeneration techniques are well known and not critical to the present invention, any technique which accomplishes the regeneration and produces fertile plants may be used.

VI. Analysis of R1 Progeny

The plants regenerated from the transformed callus are referred to as the R0 generation or R0 plants. The seeds produced by various sexual crosses of the R0 generation plants are referred to as R1 progeny or the R1 generation. When R1 seeds are germinated, the resulting plants are also referred to as the R1 generation.

To confirm the successful transmission and inheritance of the heterologous DNA in the sexual crosses described above, the R1 generation should be analyzed to confirm the presence of the transforming DNA. The analysis may be performed in any of the manners such as were disclosed above for analyzing the bombarded callus for evidence of transformation, taking into account the fact that plants and plant parts are being used in place of the callus.

VII. Breeding of Genetically Engineered Commercial Hybrid Seed

Generally, the commercial value of the transformed corn produced herein will be greatest if the heterologous DNA can be incorporated into many different hybrid combinations. A farmer typically grows several varieties of hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based upon his physical location since hybrids adapted to one part of the corn belt are generally not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the heterologous DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable heterologous DNA. This may conveniently be done by breeding programs in which a conversion process (backcrossing) is performed by crossing the initial transgeniC fertile plant to normal elite inbred lines and then crossing the progeny back to the normal parent. The progeny from this cross will segregate such that some of the plants will carry the heterologous DNA whereas some will not. The plants that do carry the DNA are then crossed again to the normal plant resulting in progeny which segregate once more. This crossing is repeated until the original normal parent has been converted to a genetically engineered line containing the heterologous DNA and also possessing all other important attributes originally found in the parent. A separate backcrossing program will be used for every elite line that is to be converted to a genetically engineered elite line. It may be necessary for both parents of a hybrid seed corn to be homozygous for the heterologous DNA. Corn breeding and the techniques and skills required to transfer genes from one line or variety to another are well-known to those skilled in the art. Thus introducing heterologous DNA into lines or varieties which do not generate the appropriate calli can be readily accomplished by these breeding procedures.

VIII. Uses of Transgenic Plants

The transgenic plants produced herein are expected to be useful for a variety of commercial and research purposes. Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g. agronomic traits such as pest resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g. improved nutritive content in human food or animal feed) or beneficial to the food processor (e.g. improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods, however, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silage or for ornamental purposes (e.g. Indian corn). Often chemical constituents (e.g. oils or starches) of corn and other crops are extracted for food or industrial use and transgenic plants may be created which have enhanced or modified levels of such components. The plants may also be used for seed production for a variety of purposes.

Transgenic plants may also find use in the commercial manufacture of proteins or other molecules encoded by the heterologous DNA contained therein, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules, or for other purposes (e.g. for research).

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded by the heterologous DNA may be transferred, e.g. from corn cells to cells of other species e.g. by protoplast fusion.

The transgenic plants may have many uses in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. The methods of the invention may also be used to create plants having unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties.

The following non-limiting examples are illustrative of the present invention. They are presented to better explain the general procedures which were used to prepare the fertile *Zea mays* plants of this invention which stably express the heterologous DNA and which transmit that DNA to progeny. All parts and percents are by weight unless otherwise specified. It must be recognized that a specific transformation event is a function of the amount of material subjected to the transfermation procedure. Thus when individual situations arise in which the procedures described herein do not produce a transformed product, repetition of the procedures will be required.

EXAMPLE I

Fertile transgenic *Zea mays* plants which contain heterologous DNA which is heritable were prepared as follows:

I. Initiation and Maintenance of Maize Cell Cultures Which Retain Plant Regeneration Capacity

Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred line A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when

the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient agar initiation/maintenance media with the root/shoot axis exposed to the medium. Initiation/maintenance media (hereinafter refered to as F medium) consisted of N6 basal media (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc. San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

The immature embryos were incubated at 26° C. in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh media. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell culture named AB12 used in this example was such a culture and had been initiated about 1 year before bombardment.

II. Plasmids—pCHNI-1, pHYGI1, pBII2.21, and pLUC-1

The plasmids pCHN1-1, pHYGI1, and pLUC-1 were constructed in the vector pBS+ (Stratagene, Inc., San Diego, Calif.), a 3.2 Kb circular plasmid, using standard recombinant DNA techniques. pCHN1-1 contains the hygromycin B phosphotransferase (HPT) coding sequence from *E. coli* (Gritz et al. 1983) flanked at the 3' end by the nopaline synthase (nos) polyadenylation sequence of *Agrobacterium tumefaciens* M. Bevan et al., *Nuc. Acids Res.*, 11, 369, 1983). Expression is driven by the cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982), located upstream from the hygromycin coding sequence. The plasmid pHYGI1 was constructed by inserting the 553 bp Bcl-BamHI fragment containing the maize AdhlS first intron (Callis et al. 1987) between the CaMV 35S promoter and the hygromycin coding sequence of pCHN1-1. A map of pHYGI1 is provided as FIG. 1A.

pBII221 contains the *E. Coli* B-glucuronidase coding sequence flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. The plasmid was constructed by inserting the maize AdhIS first intron between the 35S promoter and the coding sequence of pBI221 (Jefferson et al. 1987). A map of pBII221 is provided as FIG. 2A.

pLUC-1 contains the firefly luciferase coding sequence (DeWet et al. 1987) flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. This plasmid was used solely as a negative control.

Plasmids were introduced into the embryogenic callus culture AB12 by microprojectile bombardment.

III. DNA Delivery Process

The embryogenic maize callus line AB12 was subcultured 7 to 12 d prior to microprojectile bombardment. AB12 was prepared for bombardment as follows. Five clumps of callus, each approximately 50 mg in wet weight were arranged in a cross pattern in the center of a sterile 60 x 15 mm petri plate (Falcon 1007). Plates were stored in a closed container with moist paper towels throughout the bombardment process. Twenty six plates were prepared.

Plasmids were coated onto M-10 tungsten particles (Biolistics) exactly as described by Klein, et al (1988b) except that, (i) twice the recommended quantity of DNA was used, (ii) the DNA precipitation onto the particles was performed at 0° C., and (iii) the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process.

All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. When two plasmids were used simultaneously, each was present in an amount of 2.5 ul. One tube contained only plasmid pBII221; two tubes contained both plasmids pHYGI1 and pBII221; two tubes contained both plasmids pCHNI-1 and pBII221; and one tube contained only plasmid pLUC-1.

All tubes were incubated on ice for 10 min., pelletized by centrifugation in an Eppendorf centrifuge at room temperature for 5 seconds, and 25 ul of the supernatant was discarded. The tubes were stored on ice throughout the bombardment process. Each preparation was used for no more than 5 bombardments.

Macroprojectiles and stopping plates were obtained from Biolistics, Inc. (Ithaca, N.Y.). They were sterilized as described by the supplier. The microprojectile bombardment instrument was obtained from Biolistics, Inc.

The sample plate tray was positioned at the position 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate in the slot below the barrel. Plates of callus tissue prepared as described above were centered on the sample plate tray and the petri dish lid removed. A 7×7 cm square rigid wire mesh with 3×3 mm mesh and made of galvanized steel was placed over the open dish in order to retain the tissue during the bombardment. Tungsten/DNA preparations were sonicated as described by Biolistics, Inc. and 2.5 ul was pipetted onto the top of the macroprojectiles. The instrument was operated as described by the manufacturer. The following bombardments were performed:

2×pBII221 prep To determine transient expression frequency

10×pHYGI1/pBII221 As a potential positive treatment for transformation

1×pCHN1-1/pBII221 As a potential positive treatment for transformation

4×pLUC-1 Negative control treatment

The two plates of callus bombarded with pBII221 were transferred plate for plate to F medium (with no hygromycin) and the callus cultured at 26° C. in the dark. After 2 d this callus was then transferred plate for plate into 35×10 mm petri plates (Falcon 1008) containing 2 ml of GUS assay buffer which consists of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (Research Organics), 100 mM sodium phosphate pH 7.0, 5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM EDTA, and 0.06% Triton X-100. These were incubated at 37° C. for 3 d after which the number of blue cells was counted giving 291 and 477 transient GUS expressing cells in the two plates, suggesting that the DNA delivery process had also occurred with the other bombarded plates. These plates were discarded after counting since the GUS assay is destructive.

IV. Selection Process

Hygromycin B (Calbiochem) was incorporated into the medium by addition of the appropriate volume of filter sterilized 100 mg/ml Hygromycin B in water when the media had cooled to 45° C. prior to pouring plates.

Immediately after all samples had been bombarded, callus from all of the plates treated with pHYGI1/pBII221, pCHN1-1/pBII221 and three of the plates treated with pLUC-1 were transferred plate for plate onto F medium containing 15 mg/l hygromycin B, (five pieces of callus per plate). These are referred to as round 1 selection plates. Callus from the fourth plate treated with pLUC-1 was transferred to F medium without hygromycin. This tissue was subcultured every 2–3 weeks onto nonselective medium and is referred to as unselected control callus.

After two weeks of selection, tissue appeared essentially identical on both selective and nonselective media. All callus from eight plates from each of the pHYGI1/pBII221 and pCHN1-1/pBII-221 treatments and two plates of the control callus on selective media were transferred from round 1 selection plates to round 2 selection plates that contained 60 mg/l hygromycin. The round 2 selection plates each contained ten 30 mg pieces of callus per plate, resulting in an expansion of the total number of plates.

The remaining tissue on selective media, two plates each of pHYGI1/pBII221 and pCHN1-1/pBII221 treated tissue and one of control callus, were placed in GUS assay buffer at 37° C. to determine whether blue clusters of cells were observable at two weeks post-bombardment. After 6 d in assay buffer this tissue was scored for GUS expression.

TREATMENT	REPLICATE	OBSERVATIONS
pLUC-1		no blue cells
pHYGI1/pBII221	plate 1	11 single cells
		1 four cell cluster
	plate 2	5 single cells
pCHN1-1/pBII221	plate 1	1 single cell
		2 two cell clusters
	plate 2	5 single cells
		1 two cell cluster
		2 clusters of 8–10 cells

After 21 d on the round 2 selection plates, all viable portions of the material were transferred to round 3 selection plates containing 60 mg/l hygromycin. The round 2 selection plates, containing only tissue that was apparently dead, were reserved. Both round 2 and 3 selection plates were observed periodically for viable proliferating sectors.

After 35 d on round 3 selection plates both the round 2 and round 3 sets of selection plates were checked for viable sectors of callus. Two such sectors were observed proliferating from a background of dead tissue on plates treated with pHYGI1/pBII221. The first sector named 3AA was from the round 3 group of plates and the second sector named 6L was from the round 2 group of plates. Both lines were then transferred to F medium without hygromycin.

After 19 d on F medium without hygromycin the line 3AA grew very little whereas the line 6L grew rapidly. Both were transferred again to F medium for 9 d. The lines 3AA and 6L were then transfered to F medium containing 15 mg/l hygromycin for 14 d. At this point, line 3AA was observed to be of very poor quality and slow growing. The line 6L however grew rapidly on F medium with 15 mg.l hygromycin. In preparation for an inhibition study of the line 6L on hygromycin, the line was then subcultured to F medium without hygromycin.

After 10 d on F medium an inhibition study of the line 6L was initiated. Callus of 6L was transfered onto F medium containing 0, 10, 30, 100, and 250 mg/l hygromycin B. Five plates of callus were prepared for each concentration and each plate contained ten approximately 50 mg pieces of callus. One plate of unselected control tissue was prepared for each concentration of hygromycin.

It was found that the line 6L was capable of sustained growth over 9 subcultures on 0, 10, 30, 190, and 250 mg/l hygromycin. The name of the line 6L was changed at this time from 6L to PH1 (Positive Eygromycin transformant 1)

Additional sectors were recovered at various time points from the round 2 and 3 selection plates. None of these were able to grow in the presence of hygromycin for multiple rounds, i.e. two or three subcultures.

V. Confirmation of Transformed Callus

To show that the PH1 callus had acquired the hygromycin resistance gene, a Southern blot of PH1 callus was prepared as follows: DNA was isolated from PH1 and unselected control calli by freezing 2 g of callus in liquid nitrogen and grinding it to a fine powder which was transferred to a 30 ml Oak Ridge tube containing 6 ml extraction buffer (7M urea, 250 mM NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1% sarcosine). To this was added 7 ml of phenol:chloroform 1:1, the tubes shaken and incubated at 37° C. 15 min. Samples were centrifuged at 8K for 10 min. at 4° C. The supernatant was pipetted through miracloth (Calbiochem 475855) into a disposable 15 ml tube (American Scientific Products, C3920–15A) containing 1 ml 4.4M ammonium acetate, pH 5.2. Isopropanol, 6 ml, was added, the tubes shaken, and the samples incubated at –20° C. for 15 min. The DNA was pelleted in a Beckman TJ-6 centrifuge at the maximum speed for 5 min. at 4° C. The supernatant was discarded and the pellet was dissolved in 500 ul TE-10 (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) 15 min. at room temperature. The samples were transferred to a 1.5 ml Eppendorf tube and 100 ul 4.4M ammonium acetate, pH 5.2 and 700 ul isopropanol were added. This was incubated at –20° C. for 15 min. and the DNA pelleted 5 min. in an Eppendorf microcentrifuge (12,000 rpm). The pellet was washed with 70% ethanol, dried, and resuspended in TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The isolated DNA (10 ug) was digested with BamHI (NEB) and electrophoresed in a 0.8% w/v agarose gel at 15V for 16 hrs in TAE buffer (40 mM Tris-acetate, i mM EDTA). The DNA within the gel was then depurinated by soaking the gel twice in 0.25 M HCl for 15 min., denatured and cleaved by soaking the gel twice in 0.5M NaOH/1.0M NaCl 15 min., and neutralized by soaking the gel twice in 0.5M Tris pH 7.4/3M NaCl 30 min. DNA was then blotted onto a Nytran membrane (Shleicher & Shuell) by capillary transfer overnight in 6X SSC (20X SSC, 3M NaCl, 0.3M sodium citrate pH 7.0). The membrane was baked at 80° C. for 2 hrs under vacuum. Prehybridization treatment of the membrane was done in 6X SSC, 10X Denhardt's solution, 1% SDS, 50 ug/ml denatured salmon sperm DNA using 0.25 ml prehybridization solution per cm^2 of membrane. Prehybridization was carried out at 42° C. overnight.

A 32P labelled probe was prepared by random primer labelling with an Oligo Labelling Kit (Pharmacia) as per the suppliers instructions with 32P-dCTP (ICN Radiochemicals). The template DNA used was the 1055 bp BamHI fragment of pHYGI1, which is the HPT coding sequence. The fragment was gel purified and cut again with PsI (NEB) before labelling.

The hybridization was performed in 50% formamide, 6X SSC, 1% SDS, 50 ug/ml denatured salmon sperm DNA (Sigma), 0.05% sodium pyrophosphate and all of the isopropanol precipitated heat denatured probe (107 CPM/50ug template). The hybridization was carried out at 42° C. overnight.

The membrane was washed twice in 50 ml 6X SSC, 0.1% SDS 5 min. at room temperature with shaking, then twice in 500 ml 6X SSC, 0.1% SDS 15 min. at room temperature, then twice in 500 ml 1X SSC, 1% SDS 30 min. at 42° C., and finally in 500 ml 0.1X SSC 1% SDS 60 min. at 65° C. Membranes were exposed to Kodak X-OMAT AR film in an X-OMATIC cassette with intensifying screens. As shown in FIG. 3B, a band was observed for PH1 callus at the expected position of 1.05 Kb, indicating that the HPT coding sequence was present. No band was observed for control callus.

VI. Plant Regeneration and Production of Seed

PH1 callus was transferred directly from all of the concentrations of hygromycin used in the inhibition study to RM5 medium which consists of MS basal salts (Murashige et al. 1962) supplemented with thiamine HCl 0.5 mg/l, 2,4-D 0.75 mg/l, sucrose 50 g/l, asparagine 150 mg/l, and Gelrite 2.5 g/l (Kelco Inc. San Diego).

After 14 d on RM5 medium the majority of PH1 and negative control callus was transferred to R5 medium which is the same as RM5 medium, except that 2,4-D is omitted. These were cultured in the dark for 7 d at 26° C. and transferred to a light regime of 14 hours light and 10 hours dark for 14 d at 26° C. At this point, plantlets that had formed were transferred to one quart canning jars (Ball) containing 100 ml of R5 medium. Plants were transferred from jars to vermiculite after 14 and 21 d. Plants were grown in vermiculite for 7 or 8 d before transplanting into soil and grown to maturity. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the Ro tissue, a Southern blot was performed as previously described on leaf DNA from three randomly chosen Ro plants of PH1. As shown in FIG. 4B, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred lines A188, B73 and Oh43. Seed was harvested 45 days post-pollination and allowed to dry further 1–2 weeks. Seed set varied from 0 to 40 seeds per ear when PH1 was the female parent and from 0 to 32 seeds per ear when PH1 was the male parent.

VII. Analysis of the R1 Progeny

The presence of the hygromycin resistance trait was evaluated by a root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears.

(A) Root Elongation Bioassay

Seed was sterilized in a 1:1 dilution of commercial bleach in water plus alconox 0.1% for 20 min. in 125 ml Erlenmeyer flasks and rinsed 3 times in sterile water and imbibed overnight in sterile water containing 50 mg/ml captan by shaking at 150 rpm.

After imbibition, the solution was decanted from the flasks and the seed transferred to flow boxes (Flow Laboratories) containing 3 sheets of H_2O saturated germination paper. A fourth sheet of water saturated germination paper was placed on top of the seed. Seed was allowed to germinate 4 d.

After the seed had germinated, approximately 1 cm of the primary root tip was excised from each seedling and plated on MS salts, 20 g/l sucrose, 50 mg/l hygromycin, 0.25% Gelrite, and incubated in the dark at 26° C. for 4 d.

Roots were evaluated for the presence or absence of abundant root hairs and root branches. Roots were classified as transgenic (hygromycin resistant) if they had root hairs and root branches, and untransformed (hygromycin sensitive) if they had limited numbers of branches. The results are shown in Table 1.

(B) Etiolated leaf bioassay

After the root tips were excised as described above, the seedlings of one PH1 ear and one control ear were transferred to moist vermiculite and grown in the dark for 5 d. At this point 1 mm sections were cut from the tip of the coleoptile, surface sterilized 10 seconds, and plated on MS basal salts, 20 g/l sucrose, 2.5 g/l Gelrite with either 0 (control) or 100 mg/l hygromycin and incubated in the dark at 26° C. for 18 hr. Each plate contained duplicate sections of each shoot. They were then incubated in a light regimen of 14 hours light 10 hours dark at 26° C. for 48 hr, and rated on a scale of from 0 (all brown) to 6 (all green) for the percent of green color in the leaf tissue. Shoots were classified as untransformed (hygromycin sensitive) if they had a rating of zero and classified as transformed (hygromycin resistant) if they had a rating of 3 or greater. The results are shown in Table 1.

(C) Southern Blots

Seedling form the bioassays were transplanted to soil and are growing to sexual maturity. DNA was isolated from 0.8 g of leaf tissue after about 3 weeks and probed with the HPT coding sequence as described previously. Plants with a 1.05 Kb band present in the Southern blot were classified as transgenic. As shown in FIG. 5B, two out of seven progeny of PH1 plant 3 were transgenic as were three out of eight progeny of PH1 plant 10. The blot results correlated precisely with data from the bioassays, confirming that the heterologous DNA was transmitted through one complete sexual life cycle. All data are summarized in Table 1.

TABLE 1

	ANALYSIS OF PH1 R1 PLANTS						
PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.1	+	ND	+	4.1	–	ND	ND
3.2	–	ND	–	4.2	–	ND	ND

TABLE 1-continued

	ANALYSIS OF PH1 R1 PLANTS						
PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.3	–	ND	–	4.3	–	ND	ND
3.4	–	ND	–	4.4	–	ND	ND
3.5	–	ND	–	4.5	–	ND	ND
3.6	+	ND	+	4.6	–	ND	ND
3.7	–	ND	–	4.7	–	ND	ND
				2.1	–	ND	–
10.1	+	+	+	1.1	–	–	–
10.2	+	+	+	1.2	–	–	ND
10.3	–	–	ND	1.3	–	–	ND
10.4	–	–	–	1.4	–	–	ND
10.5	–	–	–	1.5	–	–	ND
10.6	–	–	–	1.6	–	–	ND
10.7	–	–	–	1.7	–	–	ND
10.8	ND	+	+	1.8	–	–	ND

KEY: + = transgenic; – = nontransgenic; ND = not done

EXAMPLE II

The procedure of Example I was repeated with minor modifications.

I. Plant lines and tissue cultures

The embryogenic maize callus line, AB12, was used as in Example I. The line had been initiated about 18 months before the actual bombardment occurred.

II. Plasmids

The plasmids pBII221 and pHYGI1 described in Example I were used.

III. DNA delivery process

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. One tube contained only plasmid pBII221; two tubes contained only plasmid pHYGI1; and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

2 × pBII221 prep	For transient expression
7 × pHYGI1 prep	Potential positive treatment
3 × TE prep	Negative control treatment

After all the bombardments were performed, the callus from the pBII221 treatments was transferred plate for plate to F medium as five 50 mg pieces. After 2 d the callus was placed into GUS assay buffer as per Example I. Numbers of transiently expressing cells were counted and found to be 686 and 845 GUS positive cells, suggesting that the particle delivery process had occurred in the other bombarded plates.

IV. Selection of Transformed Callus

After bombardment the callus from the pHYGI1 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate (different from Example I). The same was done for two of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 13 d the callus on round 1 selection plates was indistinguishable from unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin. An approximate five fold expansion of the numbers of plates occurred.

The callus on round 2 selection plates had increased substantially in weight after 23 d, but at this time appeared close to dead. All of the callus was transfered from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin. This transfer of all material from round 2 to round 3 selection differs from Example I in which only viable sectors were transferred and the round 2 plates reserved.

At 58 d post-bombardment three live sectors were observed proliferating from the surrounding dead tissue. All three lines were from pHYGI1 treatments and were designated 24C, 56A, and 55A.

After 15 d on maintainance medium, growth of the lines was observed. The line 24C grew well whereas lines 55A and 56A grew more slowly. All three lines were transferred to F medium containing 60 mg/l hygromycin. Unselected control callus from maintenance was plated to F medium having 60 mg/l hygromycin.

After 19 d on 60 mg/l hygromycin the growth of line 24C appeared to be entirely uninhibited, with the control showing approximately 80% of the weight gain of 24C. The line 56A was completely dead, and the line 55A was very close. The lines 24C and 55A were transferred again to F 60 mg/l hygromycin as was the control tissue.

After 23 d on 60 mg/l hygromycin the line 24C again appeared entirely uninhibited. The line 55A was completely dead, as was the negative control callus on its second exposure to to F 60 mg/l hygromycin.

At 88 d post-bombardment, a sector was observed proliferating among the surrounding dead tissue on the round 3 selection plates. The callus was from a plate bombarded with pHYGI1 and was designated 13E. The callus was transferred to F medium and cultured for 19 d. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin. After 14 d of culture, the callus line 13E

appeared uninhibited on both levels of hygromycin. The control callus appeared to have about 80% of the weight gain of 13E. The callus lines were transferred to fresh media at the same respective levels of hygromycin.

V. Confirmation of Transformed Callus

A Southern blot was prepared from DNA from the line 24C. As shown in FIG. 6B, a band was observed for the line 24C at the expected size of 1.05 Kb showing that the line 24C contained the HPT coding sequence. No band was observed for DNA from control tissue. The name of the callus line 24C was changed to PH2.

VI. Plant Regeneration and Production of Seed

The line 24C along with unselected control callus were placed onto RM5 medium to regenerate plants as in Example I. After 16 d the callus was transferred to R5 medium as in Example I.

EXAMPLE III

The procedure of Example II was repeated exactly except that different plasmids were used.

The plasmids pBII221 and pHYGI1 described in Example I were used as well as pMS533 which is a plasmid that contains the insecticidal Bacillus thuringiensis endotoxin (BT) gene fused in frame with the neomycin phosphotransferase (NPTII) gene. 5' of the fusion gene are located segments of DNA from the CaMV 35S and nopaline synthase promoters. 3' from the fusion gene are segments of DNA derived from the .tomato protease inhibitor I gene and the poly A region of the nopaline synthase gene.

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. Two tubes contained plasmids pHYGI1 and pMS533 and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

9 × pHYGI1/pMS533	Potential positive treatment
2 × TE prep	Control treatment

After bombardment the callus from the pHYGI1/pMS533 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate. The same was done for one of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 12 d the callus on round 1 selection plates appeared to show about 90% of the weight gain of the unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin as ten 30 mg pieces per plate.

After 22 d of selection on round 2 selection plates, the callus appeared completely uninhibited. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin.

At 74 d post-bombardment a single viable sector was observed proliferating from the surrounding necrotic tissue. The callus line was from pHYGI1/pMS533 treated material and was designated 86R. The callus line 86R was transferred to F medium.

After 24 d the callus line 86R had grown substantially. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin.

After 19 d of culture, the callus line 86R appeared to grow rapidly and was uninhibited on both levels of hygromycin. The control callus appeared to have only about 50% of the weight gain of 86R. The callus lines were transferred to fresh media at the same respective levels of hygromycin to further test the resistance of the callus line 86R to hygromycin.

COMPARATIVE EXAMPLE A

The basic procedures of Examples i-III have been attempted except varying the selection regime or the form of the callus. These other attempts, which are detailed in Table 2 below, were not successful. Since they were not repeated several times, it is not known whether they can be made to work. In all of the procedures, no viable sectors were observed. In the Table, "Sieved" indicates that the callus was passed through an 860 micron sieve before bombardment; the selective agent was hygromycin for each case except when pMXTI1 was the plasmid and methotrexate the selection agent.

TABLE 2

Summary of Comparative Example A

Recip. Tissue	Plasmids	Recov. Period	Round 1 Level	Round 1 Period	Round 2 Level	Round 2 Period
Clumps	pCHN1-1 pBII221	13	60	21	60	81
Clumps	pCHN1-1 pBII221	14	100	22	—	—
Clumps	pHYGI1 pBII221	8	60	19	30	132
Clumps	pCHN1-1 pBII221	0	30	22	60	109
Clumps	pMTXI1 pBII221	8	3	103	—	—
Sieved	pCHN1-1 pBII221	13	—	—	—	—

What is claimed is:

1. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, (ii) identifying or selecting a population of transformed cells, and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, and imparts herbicide resistance thereto.

2. The process of claim 1 wherein the fertile transgenic *Zea mays* plant is regenerated from transformed embryogenic tissue.

3. The process of claim 1 wherein the cells are derived from immature embryos.

4. A process comprising obtaining progeny from a fertile transgenic plant obtained by the process of claim 1 which comprise said DNA.

5. The process of claim 4 wherein said progeny are obtained by crossing said fertile transgenic plant with an inbred line.

6. The process of claim 4 comprising obtaining seed from

said progeny and obtaining further progeny plants comprising said DNA from said seed.

7. The process of claim **5** wherein the progeny obtained are crossed back to the inbred line, to obtain further progeny which comprise said DNA.

8. The process of claim **6** wherein seeds are obtained from said further progeny plants and plants comprising said DNA are recovered from said seed.

9. The process of claim **7** wherein said further progeny are crossed back to the inbred line to obtain progeny which comprise said DNA.

10. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, (ii) identifying or selecting a population of transformed cells, and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, wherein the DNA imparts insect resistance thereto.

11. The process of claim **10** wherein the fertile transgenic *Zea mays* plant is regenerated from transformed embryogenic tissue.

12. The process of claim **10** wherein the cells are derived from immature embryos.

13. A process comprising obtaining progeny from a fertile transgenic plant obtained by the process of claim **10**, which comprise said DNA.

14. The process of claim **13** wherein said progeny are obtained by crossing said fertile transgenic plant with an inbred line.

15. The process of claim **13** comprising obtaining seed from said progeny and obtaining further progeny plants comprising said DNA from said seed.

16. The process of claim **14** wherein the progeny obtained are crossed back to the inbred line, to obtain further progeny which comprise said DNA.

17. The process of claim **15** wherein seeds are obtained from said further progeny plants and plants comprising said DNA are recovered from said seed.

18. The process of claim **16** wherein said further progeny are crossed back to the inbred line to obtain progeny which comprise said DNA.

* * * * *

EXHIBIT D

US006013863A

United States Patent [19]

Lundquist et al.

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[45] Date of Patent: Jan. 11, 2000

[54] **FERTILE TRANSGENIC CORN PLANTS**

[75] Inventors: **Ronald C. Lundquist**, Minnetonka; **David A. Walters**, Bloomington, both of Minn.

[73] Assignee: **Dekalb Genetics Corporation**, Dekalb, Ill.

[21] Appl. No.: **08/844,555**

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Related U.S. Application Data

[62] Division of application No. 08/677,695, Jul. 10, 1996, which is a continuation of application No. 07/974,379, Nov. 10, 1992, Pat. No. 5,538,877, which is a continuation of application No. 07/467,983, Jan. 22, 1990, abandoned.

[51] **Int. Cl.**[7] **C12N 15/00**; C12N 15/82; A01H 1/06; A01H 4/00

[52] **U.S. Cl.** **800/293**; 800/278; 800/288; 800/300; 435/430; 435/285.3

[58] **Field of Search** ..... 800/205, 200, 800/250, DIG. 56, 293, 278, 288; 47/58, DIG. 1; 435/172.3, 172.1, 412, 424, 430, 430.1, 287, 285, 285.3

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,370,160	1/1983	Ziemelis	71/117
4,399,216	8/1983	Axel et al.	435/6
4,520,113	5/1985	Gallo et al.	436/504
4,535,060	8/1985	Comai	435/172.3
4,536,475	8/1985	Anderson	435/172.3
4,559,301	12/1985	Turner	435/76
4,559,302	12/1985	Ingolia	435/172.3
4,581,847	4/1986	Hibberd et al.	47/58
4,634,665	1/1987	Axel et al.	435/68
4,642,411	2/1987	Hibberd et al.	800/1
4,665,030	5/1987	Close	435/240
4,666,844	5/1987	Cheng	435/240
4,683,202	7/1987	Mullis	435/91
4,708,818	11/1987	Montagnier et al.	435/5
4,727,028	2/1988	Santerre et al.	435/240.2
4,743,548	5/1988	Crossway et al.	435/172.3
4,761,373	8/1988	Anderson et al.	435/172.3
4,806,483	2/1989	Wang	435/240.49
4,885,357	12/1989	Larkins et al.	530/373
4,886,878	12/1989	Larkins et al.	536/26
4,940,835	7/1990	Shah et al.	800/205
4,945,050	7/1990	Sanford et al.	435/172.1
4,956,282	9/1990	Goodman et al.	435/69.51
4,971,908	11/1990	Kishore et al.	435/172.1
5,001,060	3/1991	Peacock et al.	435/172.3
5,004,863	4/1991	Umbeck	800/205
5,013,658	5/1991	Dooner et al.	435/172.3
5,015,580	5/1991	Christou et al.	435/172.3
5,034,322	7/1991	Rogers et al.	435/172.3
5,036,006	7/1991	Sanford et al.	435/170.1
5,049,500	9/1991	Arnizen et al.	435/172.3
5,077,399	12/1991	Brauer et al.	536/27
5,082,767	1/1992	Hatfield et al.	435/6
5,094,945	3/1992	Comai	435/172.3
5,110,732	5/1992	Benfey et al.	435/172.3
5,134,074	7/1992	Gordon et al.	435/240.4

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

B-80893/87	12/1988	Australia	C12N 15/00
2032443 A1	12/1990	Canada	C12N 15/87
0 126 537 A2	4/1983	European Pat. Off. .	
0 131 623 B1	1/1984	European Pat. Off.	C12N 15/11
0 141 373 A3	5/1985	European Pat. Off.	A01G 7/00
0 142 924 A2	5/1985	European Pat. Off.	C12N 15/00
0 154 204 A2	9/1985	European Pat. Off.	C12N 15/00
0 160 390 A2	11/1985	European Pat. Off.	A01H 15/10
0 174 791 A2	3/1986	European Pat. Off.	C12N 15/00
0 189 707 A2	8/1986	European Pat. Off.	C12N 15/00
0 193 259 A1	9/1986	European Pat. Off.	C12N 15/00
0 204 549 A2	10/1986	European Pat. Off.	C12N 15/00
0 202 668 A2	11/1986	European Pat. Off.	C12N 5/02
0 242 236 A1	10/1987	European Pat. Off.	C12N 15/00
0 242 246 A1	10/1987	European Pat. Off.	C12N 15/00
0 299 552 A1	1/1988	European Pat. Off.	C12N 15/00
0 257 472 A2	3/1988	European Pat. Off.	C12N 15/00
0 262 971 A2	5/1988	European Pat. Off.	A01H 1/02
0 269 601 A2	6/1988	European Pat. Off.	C12N 15/00
0 270 356 A2	6/1988	European Pat. Off.	C12N 15/00
0 271 408 A2	6/1988	European Pat. Off.	C12N 15/00
0 275 069 A2	7/1988	European Pat. Off.	C12N 15/00

(List continued on next page.)

OTHER PUBLICATIONS

U.S. application No. 07/392,176, Adams et al., Aug. 9, 1989.
U.S. application Ser. No. 07/205,155, Entitled "Stable Transformation of Plant Cells", pp. 1–29, Filed Jun. 10, 1988.
"'Bullets' Transform Plant Cells", *Agricell Report,* 9 (Jul. 1987).
Catalog, Handbook of Fine Chemicals, Aldrich Chem. Co., p. 508 (1988).
"Cornell U. Gene Gun Hits Biotech Bullseye", *Agriculture Technology,* p. 13.
"Dalapon", Merck Index, 11th Edition, Budavae, S., (ed.), Merck and Co., pp. 405–406 (1989).
Dialog Search of Japanese Patent No. 61–134343 (1986).
EPO Notice Regarding Publication of Bibliographic Data for EPO 0485506 (1992).
"Herbicide–Resistant Corn", *CT Academy of Science and Engineering, Case Reports,* 5, 6 (1990).
International Search Report, PCT/US 90/04462, mailed Jan. 15, 1991.
International Search Report, PCT/US 94/09699, mailed Aug. 16, 1995.
Office Action dated May 30, 1989, Goldman et al., USSN 06/880,271, filed Jun. 30, 1986.
Office Action dated Mar. 8, 1990, Goldman et al., USSN 06/880,271, filed Jun. 30, 1986.
Patent Family Record for Australian Patent 8780893.

(List continued on next page.)

Primary Examiner—Gary Benzion
Attorney, Agent, or Firm—Schwegman, Lundberg, Woessner & Kluth, P.A.

[57] **ABSTRACT**

A process of preparing fertile *Zea mays* plants is provided wherein said plants are resistant to the herbicide glyphosate, as well as processes for preparing seed, human food or animal feed therefrom.

8 Claims, 6 Drawing Sheets

U.S. PATENT DOCUMENTS

5,145,777	9/1992	Goodman et al.	435/172.3
5,164,310	11/1992	Smith et al.	435/172.3
5,177,010	1/1993	Goldman et al.	435/172.3
5,187,073	2/1993	Goldman et al.	435/172.3
5,188,642	2/1993	Shah et al.	47/58
5,188,958	2/1993	Moloney et al.	435/240.4
5,196,342	3/1993	Donovan	435/320.1
5,215,912	6/1993	Hoffman	435/240.4
5,240,841	8/1993	Johnston et al.	435/172.3
5,250,515	10/1993	Fuchs et al.	514/12
5,254,799	10/1993	DeGreve et al.	800/205
5,258,300	11/1993	Glassman et al.	435/240.4
5,268,463	12/1993	Jefferson	536/23.7
5,273,894	12/1993	Strauch et al.	435/129
5,276,268	1/1994	Strauch et al.	800/205
5,278,325	1/1994	Strop et al.	554/12
5,290,924	3/1994	Last et al.	536/24.1
5,310,667	5/1994	Eichholtz et al.	435/172.3
5,350,689	9/1994	Shillito et al.	435/240.47
5,352,605	10/1994	Fraley et al.	435/240.4
5,371,003	12/1994	Murry et al.	435/172.3
5,371,015	12/1994	Sanford et al.	435/287
5,380,831	1/1995	Adang et al.	536/23.71
5,436,393	7/1995	Rocha-Sosa et al.	800/205
5,464,763	11/1995	Schilperoort et al.	435/172.3
5,484,956	1/1996	Lundquist et al.	800/205
5,489,520	2/1996	Adams et al.	435/172.3
5,495,071	2/1996	Fischhoff et al.	800/205
5,500,365	3/1996	Fischhhoff et al.	435/240.4
5,508,468	4/1996	Lundquist et al.	800/205
5,538,877	7/1996	Lundquist et al.	435/172.3
5,538,880	7/1996	Lundquist et al.	435/172.3
5,550,318	8/1996	Adams et al.	800/205
5,554,798	9/1996	Lundquist et al.	800/205
5,561,236	10/1996	Leemans et al.	800/205
5,565,347	10/1996	Fillatti et al.	435/172.3
5,567,600	10/1996	Adang et al.	536/23.71
5,567,862	10/1996	Adang et al.	800/205
5,576,203	11/1996	Hoffman	435/172.3
5,578,702	11/1996	Adang et al.	530/350
5,580,716	12/1996	Johnston et al.	435/5
5,589,616	12/1996	Hoffman et al.	800/205
5,595,733	1/1997	Carswell et al.	424/93.21
5,596,131	1/1997	Horn et al.	800/205
5,623,067	4/1997	Vanderkerckhove et al.	536/24.1
5,641,876	6/1997	McElroy et al.	536/24.1
5,693,507	12/1997	Daniell et al.	435/172.3
5,780,708	7/1998	Lundquist et al.	800/205
5,886,244	3/1999	Tomes et al.	800/293
7,392,176	8/1989	Adams et al. .	

FOREIGN PATENT DOCUMENTS

0 280 400 A2	8/1988	European Pat. Off.	A01C 1/06
0 282 164 A2	9/1988	European Pat. Off.	C12N 5/00
0 289 479 A2	11/1988	European Pat. Off.	C12N 15/00
0 290 395 A2	11/1988	European Pat. Off.	C12N 15/00
0 292 435 A1	11/1988	European Pat. Off.	C12N 15/00
0 301 749 A2	2/1989	European Pat. Off.	C12N 15/00
0 331 083 A2	9/1989	European Pat. Off.	C12N 15/00
0 331 855 A2	9/1989	European Pat. Off.	C12M 3/00
0 334 539 A2	9/1989	European Pat. Off.	C12N 15/00
0 335 528 A2	10/1989	European Pat. Off.	C12N 15/00
0 348 348 A2	12/1989	European Pat. Off.	A01N 65/00
0 442 174 A1	4/1991	European Pat. Off.	C12N 15/82
3 738 874 A1	11/1988	Germany	A01H 1/06
61-134343	5/1984	Japan .	
8 801 444	1/1990	Netherlands	C12N 15/87
2 159 173	11/1985	United Kingdom .	
85/01856	5/1985	WIPO	A01B 76/00
85/02972	7/1985	WIPO	A01C 1/06
86/01536	3/1986	WIPO	C12P 15/00
86/03776	7/1986	WIPO	C12N 15/00
87/04181	7/1987	WIPO	C12N 1/00
87/05629	9/1987	WIPO	C12N 15/00
88/08034	10/1988	WIPO	C12P 21/00
89/04371	5/1989	WIPO	C12N 21/00
89/10396	11/1989	WIPO	C12N 5/00
89/11789	12/1989	WIPO	A01H 1/00
89/12102	12/1989	WIPO	C12N 15/00

OTHER PUBLICATIONS

"Shotgunning DNA into Cells", *Genetic Engineering News,* (Jul./Aug. 1987).

Adang, M.J., et al., "Characterized Full–Length and Truncated Plasmid Clones of the Crystal Protein of *Bacillus thuringiensis* subsp. *kurstaki* HD–73 and Their Toxicity to *Manduca sexta", Gene,* 36, 289–300 (1985).

Ahokas, H., "Electrophoretic Transfection of Cereal Grains with Exogenous Nucleic Acid", Soc. Biochem. Biophys. Microbio. Fen., Biotieteen Paivat (Bioscience Days), Abstracts, Technical University of Helsinki, Espoo, p. 2 (1989).

Ahokas, H., "Transfection of Germinating Barley Seed Electrophoretically with Exogenous DNA", *Theor. Appl. Genet.,* 77, 469–472 (1989).

Altenbach, S.B., et al., "Cloning and Sequence Analysis of a cDNA Encoding a Brazil Nut Protein Exceptionally Rich in Methionine", *Plant Mol. Biol.,* 8, 239–250 (1987).

Altenbach, S.B., et al., "Enhancement of the Methionine Content of Seed Proteins by the Expression of a Chimeric Gene Encoding a Methionine–Rich Protein in Transgenic Plants", *Plant. Mol. Biol.,* 13, 513–522 (1989).

Ampe, C., et al., "The Amino–Acid Sequence of the 25 Sulphur–Rich from Seed of Brazil Nut (*Bertholletia excelsa* H.B.K.)", *Eur. J. Biochem.,* 159, 597–604 (1986).

Armstrong, C.L., et al., "Genetic and Cytogenetic Variation in Plants Regenerated from Organogenic and Friable, Embryonic Tissue Cultures of Maize", *Biol. Abstracts,* 85, Abstract 117662 (1988).

Armstrong, C.L., et al., "Establishment and Maintenance of Friable, Embryogenic Maize Callus and the Involvement of L–Proline", *Planta,* 164, 207–214 (1985).

Barker, R.F., et al., "Nucleotide Sequence of the T–DNA Region from the *Agrobacterium tumefaciens* Octopone Ti Plasmid pTi5955", *Plant Mol. Biol.,* 2, 335–350 (1983).

Benner, M.S., et al., "Genetic Analysis of Methionine–Rich Storage Protein Accumulation in Maize", *Theor. Appl. Genet.,* 78, 761–767 (1989).

Bevan, M., et al., "A Chimaeric Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation", *Nature,* 304, 184–187 (1983).

Bevan, M., et al., "Structure and Transcription of the Nopaline Synthase Gene Region of T–DNA", *Nuc. Acids Res.,* 11, 369–385 (1983).

Binns, A.N., "Agrobacterium–Mediated Gene Delivery and the Biology of Host Range Limitations", *Physiol. Plant.,* 79, 135–139 (1990).

Booy, G., et al., "Attempted Pollen–Mediated Transformation of Maize", *J. Plant Physiol.,* 135, 319–324 (1989).

Botterman, J., et al., "Engineering Herbicide Resistance in Plants," *Trends in Genetics,* 4, 221–222 (Aug. 1988).

Boulton, M.I., et al., "Specificity of Agrobacterium–Mediated Delivery of Maize Streak Virus DNA to Members of the Gramineae", *Plant Mol. Biol.,* 12, 31–40 (1989).

Boyer, J.S., "Water Deficits and Photosynthesis", In: *Water Deficits and Plant Growth,* vol. IV, Kozlowski, T.T., (ed.), Academic Press, New York, 153–190 (1976).

Brill, W.J., "Agricultural Microbiology", *Scientific American,* 245, 199–215 (Sep., 1981).

Buchanan–Wollaston, V., et al., "Detoxification of the Herbicide Dalapon by Transformed Plants", *J. Cell. Biochem.,* 13D, 330, Abstract No. M503 (1989).

Callis, J., et al., "Introns Increase Gene Expression in Cultured Maize Cells", *Genes and Development,* 1, 1183–1200 (1987).

Cao, J., et al., "Transformation of Rice and Maize Using the Biolistic Process", In: *Plant Gene Transfer,* Lamb, C.J., et al., (eds.), Alan R. Liss, Inc., New York, 21–33, (1990).

Carpita, N.C., "The Biochemistry of "Growing" Cells Walls", In: *Physiology of Cell Expansion During Plant Growth,* Cosgrove, D.J., et al., (eds.), Am. Soc. Plant Physiologists, 28–100 (1987).

Chandler, V.L., et al., "Two Regulatory Genes of the Maize Anthocyanin Pathway are Homologous: Isolation of B Utilizing R Genomic Sequences", *The Plant Cell,* 1, 1175–1183 (1989).

Charest, P.J., et al., "Factors Affecting the Use of Chloramphenicol Acetyltransferase as a Marker for Brassica Genetic Transformation", *Plant Cell Reports,* 7, 628–631 (1989).

Chourey, P.S., et al., "Callus Formation from Protoplasts of a Maize Cell Culture", *Theor. Appl. Genet.,* 59, 341–344 (1981).

Christou, P., et al., "Cotransformation Frequencies of Foreign Genes in Soybean Cell Cultures", *Theor. Appl. Genet.,* 79, 337–341 (1990).

Christou, P., et al., "Opine Synthesis in Wild–Type Plant Tissue", *Plant Physiol.,* 82, 218–221 (1986).

Christou, P., et al., "Soybean Genetic Engineering—Commercial Production of Transgenic Plants", *Trends in Biotechnol.,* 8, 145–151 (1990).

Christou, P., et al., "Stable Transformation of Soybean Callus by DNA–Coated Gold Particles," *Plant Physiol.,* 87, 671–674 (1988).

Chu, C.–C., et al., "Establishment of an Efficient Medium for Anther Culture of Rice through Comparative Experiments on the Nitrogen Sources", *Sci. Sin.* (*Peking*), 13, 659–668 (1975).

Cocking, F., et al., "Gene Transfer in Cereals", *Science,* 236, 1259–1262 (1987).

Coe, E.H., et al., "The Genetics of Corn", In: *Corn and Corn Improvement,* 2nd Edition, Sprague, G.F., (ed.), Am. Soc. Agronomy, Inc., Madison, WI, p. 138 (1977).

Comai, L., et al., "Expression in Plants of a Mutant aroA Gene from *Salmonella typhimurium* Confers Tolerance to Glyphosate", *Nature,* 317, 741–744 (Oct. 1985).

Creissen, G., et al., "Agrobacterium– and Microprojectile–Mediated Viral DNA Delivery into Barely Microspore–Derived Cultures", *Plant Cell Rep.,* 8, 680–683 (Apr. 1990).

Crossway, A., et al., "Integration of Foreign DNA Following Microinjection of Tobacco Mesophyll Protoplasts", *Mol. Gen. Genet.,* 202, 179–185 (1986).

Darvill, A., et al., "The Primary Cell Walls of Flowering Plants", In: *The Biochemistry of Plants,* vol. 1, Academic Press, Inc., New York, 91–162 (1980).

Dauce–Lereverand, B., et al., "Improvement of *Escherichia coli* Strains Overproducing Lysine Using Recombinant DNA Techniques", *Euro. J. Appl. Microbiol. and Biotechnol.,* 15, 227–231 (1982).

De Block, M., et al., "Engineering Herbicide Resistance on Plants by Expression of a Detoxifying Enzyme", *EMBO J.,* 6, 2513–2518 (1987).

De Greef, W., et al., "Evaluation of Herbicide Resistance in Transgenic Crops under Field Conditions", *Bio/Technol.,* 7, 61–64 (1989).

Dekeyser, R.A., et al., "Evaluation of Selectable Markers for Rice Transformation", *Plant Physiol.,* 90, 217–223 (1989).

Dekeyser, R.A., et al., "Transient Gene Expression in Intact and Organized Rice Tissues", *The Plant Cell,* 2, 591–602 (1990).

Dewald, S.G., et al., "Plant Regeneration from Inbred Maize Suspensions", VIIth Intl. Cong. on Plant Tissue and Cell Culture, p. 12, Abstract No. A1–36 (Jun. 24–29, 1990).

Dewet, J.M.J., et al., "Exogenous Gene transfer in Maize (*Zea mays*) Using DNA–Treated Pollen", In: *The Experimental Manipulation of Ovule Tissues,* Chapman, G.P., et al., (eds.), Longman, New York, 197–209 (1985).

Dewet, J.R., et al., "Cloning of Firefly Luciferase cDNA and the Expression of Active Luciferase in *Escherichia coli", Proc. Natl. Acad. Sci. USA,* 82, 7870–7873 (1985).

Evans, D.A., et al., "Somaclonal Variation—Genetic Basis and Breeding Applications", *Trends in Genet.,* 5, 46–50 (1989).

Fransz, P., et al., "Cytodifferentiation During Callus Initiation and Somatic Embryogenesis in *Zea mays* L.", Ph.D. Thesis, Univ. of Wageningen Press, The Netherlands (1988).

Freeling, J.C., et al., "Development Potentials of Maize Tissue Cultures", *Maydica,* XXI, 97–112 (Jul. 1977).

Freiberg, B., "More Researchers Discover Corn Transformation Technology", *AG Biotechnology News,* p. 26 (1990).

Fromm, M., et al., "Expression of Genes Transfected into Monocot and Dicot Plant Cells by Electroporation", *Proc. Nat. Acad. Sci. USA,* 82, 5824–5828 (1985).

Fromm, M.E., et al., "Stable Transformation of Maize after Gene Transfer by Electroporation", *Nature,* 319, 791–793 (1986).

Fry, S.C., "Introduction to the Growing Cell Wall", In: *The Growing Plant Cell Wall: Chemical and Metabolic Analysis,* Longman Scientific and Technical, New York, pp. 1–5, 102–109 (1988).

Geiser, M., et al., "The Hypervariable Region on the Genes Coding for Entomopathogenic Crystal Proteins of *Bacillus thuringiensis:* Nucleotide Sequence of the kurhd1 gene of subsp. kurstaki HD1", *Gene,* 48, 109–118 (1986).

Goff, S.A., et al., "Plant Regeneration of Anthocyanin Biosynthetic Genes Following Transfer of B Regulatory Genes into Maize Tissues", *EMOB J.,* 9, 2517–2522 (1990).

Gordon–Kamm, W.J., et al., "Stable Transformation of Embryonic Maize Cultures by Microprojectile Bombardment", *J. Cell. Biochem.,* 13D, p. 259, Abstract No. M122 (1989).

Gould, O., et al., "Shoot Tip Culture as a Potential Transformation System", Abstracts, Beltwide Cotton Production Research Conferences, New Orleans, LA, p. 91 (1988).

Graves, A., et al., "The transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens", Plant Mol. Biol.,* 7, 43–50 (1986).

Green, C., et al., "Plant Regeneration in Tissue Cultures of Maize", In: *Maize for Biological Research,* Sheridan, W.F., (ed.), Plant Mol. Biol. Assoc., pp. 367–372 (1982).

Green, C., et al., "Plant Regeneration from Tissue Cultures of Maize", *Crop Sci.,* 15, 417–421 (1975).

Green, C., et al., "Somatic Cell Genetic Systems in Corn", In: *Advances in Gene Technology: Molecular Genetics of Plants and Animals,* Academic Press, Inc., New York, pp. 147–157 (1983).
Grimsley, N., et al., "DNA Transfer from Agrobacterium to *Zea mays* or Brassica by Agroinfection is Dependent on Bacterial Virulence Functions", *Mol. Gen. Genet.,* 217, 309–316 (1989).
Gritz, L., et al., "Plasmid–Encoded Hygromycin B Resistance: The Sequence of Hygromycin B Phosphotransferase Gene and Its Expression in *Escherichia coli* and *Saccharomyces cerevisiae", Gene,* 25, 179–188 (1983).
Guerineau, F., et al., "Sulfonamide Resistance gene for Plant Transformation", *Plant Mol. Biol.,* 15, 127–136 (1990).
Guilley, H., et al., "Transcription of Cauliflower Mosaic Virus DNA: Detection of Promoter Sequences, and Characterization of Transcripts", *Cell,* 30, 763–773 (Oct. 1982).
Hallauer, A.R., et al., "Corn Breeding", In: *Corn and Corn Improvement,* 3rd Edition, Sprague, G.F., et al., (eds.), Agronomy Soc. Am., pp. 463–564 (1988).
Haughn, G.W., "Transformation with a Mutant Arabidopsis Acetolactate Synthase Gene Renders Tobacco Resistant to Sulfonylurea Herbicides", *Mol. Gen. Genet.,* 211, 266–271 (1988).
Hauptmann, R.M., et al., "Evaluation of Selectable Markers for Obtaining Stable Tranformants on the Gramineae", *Plant Physiol.,* 86, 602–606 (1988).
Herrera–Estrella, L., et al., "Use of Reporter Genes to Study Gene Expression in Plant Cells", In: *Plant Molecular Biology Manual B1,* Kluwer Academic Publishers, Dordrecht, pp. 1–22 (1988).
Hoffman, L.M., et al., "A Modified Storage Protein is Synthesized, Processed, and Degraded in the Seeds of Transgenic Plants", *Plant Mol. Biol.,* 11, 717–729 (1988).
Hoffman, L.M., et al., "Synthesis and Protein Body Deposition of Maize 15kD Zein in Transgenic Tobacco Seeds", *EMBO J.,* 6, 3213–3221 (1987).
Hofte, H., et al., "Insecticidal Crystal Proteins of *Bacillus thuringiensis", Microbiol. Rev.,* 53, 242–255 (1989).
Hooykaas, P.J., et al., "Transformation of Plant Cell via Agrobacterium", *Plant Mol. Biol.,* 13, 327–336 (1989).
Hooykaas–Van Slogteren, G.M.S., et al., "Expression of Ti Plasmid Genes in Monocotyledonous Plants Infected with *Agrobacterium tumefaciens", Nature,* 311, 763–764 (Oct. 25, 1984).
Horn, M., et al., "Transgenic Plants of Orchard Grass (*Dactylis glomerata* L.). from Protoplasts", *Chem. Abstracts,* 110, Abstract No. 89869a, 208 (1989).
Horn, M., et al., "Transgenic Plants of Orchardgrass (*Dactylis glomerata* L.) from Protoplasts", *Plant Cell Reports,* 7, 469 (1988).
Huang, Y., et al., "Factors Influencing Stable Transformations of Maize Protoplasts by Electroporation", *Plant Cell, Tissue and Organ Culture,* 18, 281 (1989).
Imbrie–Milligan, C., et al., "Microcallus Growth from Maize Protoplasts", *Planta,* 171, 58–64 (1987).
Jefferson, R., "Assayig Chimeric Genes in Plants: the GUS Gene Fusion System", *Plant Mol. Biol. Rep.,* 5, 387–405 (1987).
Jefferson, R., et al., "β–Glucuronidase from *Escherichia coli* as a Gene–Fusion Marker", *Proc. Nat. Acad. Sci. USA,* 83, 8447–8451 (1986).
Jefferson, R., et al., "GUS Fusions: β–Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher Plants", *EMBO J.,* 6, 3901–3907 (1987).
Johri, M.M., et al., "Genetic Approaches to Meristem Organization", In: *Maize for Biological Research,* Sheridan, W.F., (ed.), Plant Mol. Biol. Assoc., pp. 301–310 (1982).
Jones, H., et al., "Recent Advances in Plant Electroporation", *Oxford Surveys of Plant Mol. and Cell Biol.,* 4, 347–357 (1987).
Jones, H., et al., "Transient Gene Expression in Electroporated Solanum Protoplasts", *Plant Mol. Biol.,* 13, 503–511 (1989).
Kaeppler, H.F., et al., "Silicon Carbide Fiber–Mediated DNA Delivery into Plant Cells", *Plant Cell Rep.,* 9, 415–418 (1990).
Kamo, K., et al., "Establishment and Characterization of Long–Term Embryonic Maize Callus and Cell Suspension Cultures", *Plant Sci.,* 45, 111–117 (1986).
Kamo, K., et al., "Regeneration of *Zea mays* L. from Embryogenic Callus", *Bot. Gaz.,* 146, 327–334 (1985).
Kao, K.N., et al., "Nutritional Requirements for Growth of *Vicia hajastana* Cells and Protoplasts at a Very Low Population Density in Liquid Media", *Planta,* 126, 105–110 (1978).
Kartha, K., et al., "Transient Expression of Chloramphenicol Acetyl Transferase (CAT) Gene in Barley Cell Cultures and Immature Embryos Through Microprojectile Bombardment", *Plant Cell. Rep.,* 8, 429–432 (1989).
Kay, R., et al., "Duplication of CaMV 35S Promoter Sequences Creates a Strong Enhancer for Plant Genes", *Science,* 236, 1299–1302 (Jun. 5, 1987).
Kirihara, J., et al., "Differential Expression of a Gene for a Methionine–Rich Storage Protein in Maize", *Mol. Gen. Genet.,* 211, 477–484 (1988).
Kirihara, J., et al., "Isolation and Sequence of a Gene Encoding a Methionine–Rich 10–kD Zein Protein from Maize", *Gene,* 71, 359–370 (1988).
Klein, T., et al., "Genetic Transformation of Maize Cells by Particle Bombardment", *Plant Physiol.,* 91, 440–444 (1989).
Klein, T., et al., "Regulation of Anthocyanin Biosynthetic Genes Introduced into Intact Maize Tissue by Microprojectiles", *Proc. Nat. Acad. Sci. USA,* 86, 6682–6685 (1989).
Klein, T., et al., "Genetic Transformation of Maize Cells by Particle Bombardment and the Influence of Methylation on Foreign Gene Expression", In: *Gene Manipulation in Plant Improvement II,* Gustafson, J.P., (ed.), Plenum Press, New York, 265–266 (1990).
Klein, T., et al., "Transfer of Foreign Genes into Intact Maize Cells with High–Velocity Microprojectiles", *Proc. Nat. Acad. Sci. USA,* 85, 4305–4309 (1988).
Klein, T.M., et al., "Factors Influencing Gene Delivery into *Zea mays* Cells by High Velocity Microprojectiles", *Bio/Technol.,* 6, 559–563 (1988).
Klein, T.M., et al., "High Velocity Microprojectiles for Delivering Nucleic Acids to Living Cells", *Nature,* 327, 70–73 (1987).
Kozac, M., "Complication and Analysis of Sequence from the Translation Start Site in Eukaryotic mRNAs", *Nuc. Acids Res.,* 12, 857–871 (1984).
Kozac, M., "Point Mutations Define a Sequence Flanking the AUG Initiator Codon that Modulates Translation by Eukaryotic Ribosomes", *Cell,* 44, 283–292 (1986).
Kuhlemeier, C., et al., "Regulation of Gene Expression in Higher Plants", *Ann. Rev. Plant Physiol.,* 38, 234–239 (1987).
Lazzeri, P., et al., "In Vitro Genetic Manipulation of Cereals and Grasses", *Ad. Cell Culture,* 6, 291–293 (1988).

Lee, J.S., et al., "Gene Transfer into Intact Cells of Tobacco by Electroporation", *Korean J. Genet.,* 11, 65–72 (1989).

Levitt, J., "Growth Regulators", In: *Introduction to Plant Physiology,* The C.V. Mosby Company, St. Louis, p. 241 (1969).

Lindsey, K., et al., "Electroporation of Cells", *Physiol. Plant.,* 79, 168–172 (1990).

Lindsey, K., et al., "Stable Transformation of Sugarbeet Protoplasts by Electroporation", *Plant Cell Rep.,* 8, 71–74 (1989).

Lindsey, K., et al., "The Permeability of Electroporated Cells and Protoplasts of Sugar Beet", *Planta,* 172, 346–355 (1987).

Lindsey, K., et al., "Transient Gene Expression in Electroporated Protoplasts and Intact Cells of Sugar Beet", *Plant Mol. Biol.,* 10, 43–52 (1987).

Lorz, H., et al., "Advances in Tissue Cultures and Progress Towards Genetic Transformation of Cereals", *Plant Breeding,* 100, 1–25 (1988).

Lu, C., et al., "Improved Efficiency of Somatic Embryogenesis and Plant Regeneration on Tissue Cultures of Maize(*Zea mays* L.)", *Theor. Appl. Genet.,* 66, 285–289 (1983).

Lu, C., et al., "Somatic Embryogenesis in *Zea mays* L.", *Theor. Appl. Genet,* 62, 109–112 (1982).

Ludwig, S., et al., "A Regulatory Gene as a Novel Visible Marker for Maize Transformation", *Science,* 247, 449–450 (1990).

Ludwig, S., et al., "High Frequency Callus Formation from Maize Protoplasts", *Theor. Appl. Genet.,* 71, 344–350 (1985).

Ludwig, S., et al., "Lc, a Member of the Maize R Gene Family Responsible for Tissue–Specific Anthocyanin Production, Encodes a Protein Similar to Transcriptional Activators and Contains the myc–Homology Region", *Proc. Nat. Acad. Sci. USA,* 86, 7092–7096 (1989).

Ludwig, S., et al., "Maize R Gene Family: Tissue–Specific Helix–Loop–Helix Proteins", *Cell,* 62, 849–851 (1990).

Lutcke, H., et al., "Selection of AUG Initiation Codons Differs in Plants and Animals", *EMBO J.,* 6, 43–48 (1987).

Mariani, C., et al., "Engineered Male Sterility in Plants", *Symposia of the Society for Experimental Biology,* Number XLV, Proceedings of a Meeting Held at the University of Glasgow, Scotland, 271–279 (1991).

Masumura, T., et al., "cDNA Cloning of an mRNA Encoding a Sulfur–Rich 10 kDa Prolamin Polypeptide in Rice Seeds", *Plant Mol. Biol.,* 12, 123–130 (1989).

McCabe, D.E., et al., "Stable Transformation of Soybean (*Glycine max*) by Particle Acceleration", *Bio/Technol.,* 6, 923–926 (1988).

McDaniel, C., et al., "Cell–Lineage Patterns in the Shoot Apical Meristem of the Germinating Maize Embryo", *Planta,* 175, 13–22 (1988).

Meadows, M., "Characterization of Cells and Protoplasts of the B73 Maize Cell Line", *Plant Sci. Lett.,* 28, 337–348 (1982/83).

Mendel, R., et al., "Delivery of Foreign Genes to Intact Barley Cell by High–Velocity Microprojectiles", *Theor. Appl. Genet.,* 78, 31–34 (1989).

Messing, J., "Corn Storage Protein: A Molecular Genetic Model", Division of Energy BioSciences—Summaries of FY 1990 Activities, p. 70, Abstract No. 135 (1990).

Milborrow, B.V., "Abscisic Acid and Other Hormones", In: *The Physiology and Biochemistry of Drought Resistance in Plants,* Paleg, L.G., et al., (eds.), Academic Press, Inc., New York, pp. 347–388 (1981).

Morikawa, H., et al., "Gene Transfer into Intact Plant Cells by Electroporation through Cell Walls and Membranes", *Gene,* 41, 121 (1986).

Morocz, S., et al., "An Improved System to Obtain Fertile Regenerants via Maize Protoplasts Isolated from a Highly Embryonic Suspension Culture", *Theor. Appl. Genet.,* 80, 721–726 (1990).

Morocz, S., et al., "Two Approaches to Rendering *Zea mays* L. Applicable to Tissue Culture Manipulations", Abstracts, VIIth Intl. Cong. on Plant Tissue and Cell Culture, Amsterdam, A1–102, Abstract No. 209, p. 190 (1990).

Murakami, T., et al., "The Bialaphos Biosynthetic Genes of *Streptomyces hygroscopicus:* Molecular Cloning and Characterization of the Gene Cluster", *Mol. Gen. Genet.,* 205, 42–50 (1986).

Murashige, T., et al., "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures", *Physiol. Plant.,* 15, 473–497 (1962).

Murphy, H.L., "New Dekalb–Pfizer Seed Chief to Harvest R & D Breakthroughs", *Crain's Business Weekly,* pp. 38–39 (1990).

Murray, E.E., et al., "Codon Usage in Plant Genes", *Nuc. Acids Res.,* 17, 477–498 (1989).

Nelson, R.S., "Virus Tolerance, Plant Growth, and Field Performance of Transgenic Tomato Plants Expressing Coat Protein from Tobacco Mosaic Virus", *Bio/Technol.,* 6, 403–409 (1988).

Nelson, T., "New Horses for Monocot Gene Jockeys", *The Plant Cell,* 2, 589 (Jul., 1990).

Neuffer, M.G., "Growing Maize for Genetic Purposes", Maize for Biological Research, Plant Mol. Biol. Assoc., pp. 19–30 (1988).

Odell, J., et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter", *Nature,* 313, 810–811 (1985).

Ohta, Y., et al., "High–Efficiency Genetic Transformation of Maize by a Mixture of Pollen and Exogenous DNA", *Pro. Nat. Acad. Sci. USA,* 83, 715–719 (1986).

Okta, Y., et al., "Gene Manifestation of Exogenous DNA Applied to Self–Propagating Stigma (Gene Action Revealed in the M1 and M2 Generations from Self–Pollination Applying Exogenous DNA", *Jap. J. Breed.,* 30, 184–185 (1980).

Ozias–Akins, P., et al., "In vitro Regeneration and Genetic Manipulation of Grasses", *Physiol. Plant.,* 73, 565–569 (1988).

Ozias–Akins, P., et al., "Progress and Limitations in the Culture of Cereal Protoplasts", *Trends in Biotechnol.,* 2, 119–123 (1984).

Parker, W.B., et al., "Selection and Characterization of Sethoxydim–Tolerant Maize Tissue Cultures", *Plant Physiol.,* 92, 1220–1225 (1990).

Pederson, K., et al., "Sequence Analysis and Characterization of a Maize Gene Encoding a High–Sulfur Zein Protein of M_1 15,000", *J. Biol. Chem.,* 261, 6279–6284 (1986).

Phillips, R.L., et al., "Cell/Tissue Culture and In Vitro Manipulation", In: *Corn and Corn Improvement,* 3rd Edition, Sprague, G.F., et al., (eds.), Agronomy Soc. Am., pp. 345–387 (1988).

Phillips, R.L., et al., "Elevated Protein–Bound Methionine in Seeds of a Maize Line Resistant to Lysine Plus Threonine", *Cereal Chem.,* 62, 213–218 (1985).

Poehlman, J., "Breeding Corn (Maize)", In: *Breeding Field Crops,* 3rd Edition, AVI Publishing Co., Westport, CT, pp. 452, 469–471, 477–481 (1986).

Potrykus, I., "Gene Transfer to Cereals: An Assessment" *Bio/Technol.,* 8, 535–542 (Jun. 1990).

Potrykus, I., "Gene Transfer to Cereals: An Assessment", *Trends Biotechnol.,* 7, 269–273 (Oct. 1989).

Potrykus, I., "Gene Transfer to Plants: Assessment and Perspectives", *Physiol. Plant.,* 79, 125–134 (1990).

Potrykus, I., et al., "Callus Formation from Cell Culture Protoplasts of Corn (*Zea mays* L.)", *Theor. Appl. Genet. 54,* 209–214 (1979).

Potrykus, I., et al., "Callus Formation from Stem Protoplasts of Corn (*Zea mays* L.)", *Mol. Gen. Genet.,* 156, 347–350 (1977).

Potter, H. et al., "Enhancer–Dependent Expression of Human κ Immunoglobulin Genes Introduced into Mouse Pre–B Lymphocytes by Electroporation", *Pro. Nat. Acad. Sci. USA,* 81, 7161–7165 (1984).

Prioli, L.M., et al., "Plant Regeneration and Recovery of Fertile Plants from Protoplasts of Maize (*Zea mays* L.)", *Bio/Technol.,* 7, 589–594 (Jun. 1989).

Puite, K.J., et al., "Electrofusion, a Simple and Reproducible Technique in Somatic Hybridization of *Nicotiana plumbaginifolia* Mutants", *Plant Cell Rep.,* 4, 274–276 (1985).

Ranch, J.P., et al., "Expression of 5–Methyltryptophan Resistance in Plants Regenerated from Resistant Cell Lines of *Datura innoxia", Plant Physiol.,* 71, 136–140 (1983).

Rhodes, C.A., "Corn: from Protoplasts to Fertile Plants", *Bio/Technol.,* 7, 548 (Jun. 1989).

Rhodes, C.A., "Genetically Transformed Maize Plants from Protoplasts", *Science,* 240, 204–207 (Apr. 8, 1988).

Rhodes, C.A., et al., "Plant Regeneration from Protoplasts Isolated from Embryogenic Maize Cell Cultures", *Bio/Technol.,* 6, 56–60 (Jan. 1988).

Richaud, F., et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli* dapA Gene", *Biol. Abstracts,* 82, p. AB–391, Abstract No. 3396 (1986).

Richaud, F., et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli* dapA Gene", *J. Bacteriol.,* 166, 297–300 (1986).

Robertson, D.S., "Loss of Mu Mutator Activity When Active Mu Systems are Transferred to Inbred Lines", *Maize Genetics Coop. Newsletter,* 60, 10 (1986).

Ross, M.C., et al., "Transient and Stable Transgenic Cells and Calli of Tobacco and Maize Following Microprojectile Bombardment", *J. Cell. Biochem.,* 13D, p. 268, Abstract No. M149 (1989).

Sahi, S.V., et al., "Metabolites in Maize Which Affect Virulence Induction in *Agrobacterium tumefaciens", Plant Physiol., Supplement,* p. 86, Abstract No. 514 (1989).

Sanford, J.C., "Biolistic Plant Transformation", *Physiol. Plant.,* 79, 206–209 (1990).

Sanford, J.C., "The Biolistic Process", *Trends Biotechnol.,* 6, 299–302 (1988).

Sanford, J.C., et al., "Attempted Pollen–Mediated Plant Transformation Employing Genomic Donor DNA", *Theor. Appl. Genet.,* 69, 571–574 (1985).

Sanford, J.C., et al., "Delivery of Substances into Cells and Tissues Using a Particle Bombardment Process", *Particulate Sci. Technol.,* 5, 27–37 (1987).

Sass, J.E., "Morphology: Development of the Caryopsis", In: *Corn and Corn Improvement,* 2nd Edition, Sprauge, G.F., (ed.), Am. Soc. Agronomy, pp. 89, 98 (1977).

Schmidt, A., et al., "Media and Environmental Effects of Phenolics Production from Tobacco Cell Cultures", *Chem. Abstracts.,* 110, p. 514, Abstract No. 230156z (1989).

Shigekawa, K., et al., "Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells", *BioTechniques,* 6, 742–751 (1988).

Shillito, R.D., et al., "High Efficiency Direct Gene Transfer to Plants", *Bio/Technol.,* 3, 1099 (1985).

Shillito, R.D., et al., "Regeneration of Fertile Plants from Protoplasts of Elite Inbred Maize", *Bio/Technol.,* 7, 581–587 (Jun. 1989).

Shimamoto, K., et al., "Fertile Transgenic Rice Plants Regenerated from Transformed Protoplasts", *Nature,* 338, 274–278 (1989).

Shotwell, M.A., et al., "The Biochemistry of Plants—A Comprehensive Treatise", In: *The Biochemistry of Plants,* vol. 15, Marcus, A., (ed.), Academic Press, Inc., San Diego, pp. 297–345 (1989).

Smith, R., et al., "Shoot Apex Explant for Transformation", *Plant Physiol. (Suppl.),* 86, p. 108, Abstract 646 (1988).

Soberon, X., et al., "Construction and Characterization of New Cloning Vehicles, IV. Deletion Derivatives of pBR322 and pBR325", *Gene,* 9, 287–305 (1980).

Spencer, T.M., et al., "Bialaphos Selection of Stable Transformations from Maize Cell Culture", *Theor. Appl. Genet.,* 79, 625–631 (May 1990).

Spencer, T.M., et al., "Fertile Transgenic Maize", Abstracts, 7th Annual Meeting, Mid–Atlantic Plant Mol. Biol. Soc., p. 30 (1990).

Spencer, T.M., et al., "Segregation of Transgenes in Maize", *Plant Mol. Biol.* 18, 201–210 (1992).

Spencer, T.M., et al., "Selection of Stable Transformants from Maize Suspension Cultures Using the Herbicide Bialaphos", Poster Presentation, FASEB Plant Gene Expression Conference, Copper Mountain, Colorado (Aug. 8, 1989).

Sprague, G.F., et al., "Corn Breeding", In: *Corn and Corn Improvement,* Sprague, G. F., (ed.), Am. Soc. Agronomy, Inc., Madison, WI, pp. 305, 320–323 (1977).

Sugiyama, M., et al., "Use of the Tyrosinase Gene from Streptomyces to Probe Promoter Sequences for *Escherichia coli", Plasmid,* 23, 237–241 (1990).

Thompson, C., et al., "Characterization of the Herbicide–Resistance Gene bar from *Streptomyces hygroscopicus", EMBO J.,* 6, 2519–2523 (1987).

Tomes, D.T., et al., "Transgenic Tobacco Plants and Their Progeny Derived by Microprojectile Bombardment of Tobacco Leaves", *Plant Mol. Biol.,* 14, 261–268 (Feb. 1990).

Twell, D., et al., "Transient Expression of Chimeric Genes Delivered into Pollen by Microprojectile Bombardment", *Plant Physiol.,* 91, 1271–1274 (1989).

Ulian, E., et al., "Transformation of Plants via the Shoot Apex", *In Vitro Cell. Dev. Biol.,* 9, 951–954 (1988).

Usami, S., et al., "Absence in Monocotyledonous Plants of the Diffusable Plant Factors including T–DNA Circularization and vir Gene Expression in Agrobacterium", *Mol. Gen. Genet.,* 209, 221–226 (1987).

Vasil, I.K., et al., "Culture of Protoplasts Isolated from Embryogenic Cell Suspension Cultures of Sugarcane and Maize", *IAPTC Abstracts,* p. 443 (1986).

Vasil, I.K., et al., "Isolation and Maintenance of Embryogenic Cell Suspension Cultures of Gramineae", In: *Cell Culture and Somatic Cell Genetics of Plants,* vol. I, Academic Press, Inc., pp. 152–158 (1984).

Vasil, V., et al., "Plant Regeneration from Friable Embryonic Callus and Cell Suspension Cultures of *Zea mays* L.", *J. Plant Physiol.,* 124, 399–408 (1986).
Walbot, V., et al., "Molecular Genetics of Corn", In: *Corn and Corn Improvement,* 3rd Edition, Sprague, G.F., et al., (eds.), Am. Soc. Agronomy, Madison, WI, pp. 389–430 (1988).
Waldron, C., et al., "Resistance to Hygromycin B", *Plant Mol. Biol.,* 5, 103–108 (1985).
Wang, Y., et al., "Transient Expression of Foreign Genes in Rice, Wheat and Soybean Cells Following Particle Bombardment", *Plant Mol. Biol.,* 11, 433–439 (1988).
Weising, K., et al., "Foreign Genes in Plants: Transfer, Structure, Expression and Applications", *Ann. Rev. Genet.,* 22, 421–478 (1988).
White, J., et al., "A Cassette Containing the bar Gene of *Streptomyces hygroscopicus:* a Selectable Marker for Plant Transformation", *Nuc. Acid. Res.,* 18, 1062 (1989).
Whiteley, H.R., et al., "The Molecular Biology of Parasporal Crystal Body Formation in *Bacillus thuringiensis", Ann. Rev. Microbiol.,* 40, 549–576 (1986).
Yang, H., et al., "Production of Kanamycin Resistant Rice Tissues Following DNA Uptake into Protoplasts", *Plant Cell Rep.,* 7, 421 (1988).
Yanisch–Perron, L., et al., "Improved M13 Phage Vectors and Host Strains: Nucleotide Sequences of the M13mp18 and pUC19 Vectors", *Gene,* 33, 103–119 (1985).
Yugari, Y., et al., "Coordinated End–Product Inhibition in Lysine Synthesis in *Escherichia coli", Biochem. Biophys. Acta.,* 62, 612–614 (1962).
Abe, K., et al., "Molecular Cloning of a Cysteine Proteinase Inhibitor of Rice (Oryzacystatin)", *The Journal of Biological Chemistry,* 262, 16793–16797 (Dec. 15, 1987).
Anderson, J.M., et al., "The Encoded Primary Sequence of a Rice Seed ADP–glucose Pyrophosphorylase Subunit and Its Homology to the Bacterial Enzyme", *The Journal of Biological Chemistry,* 264, 12238–12242 (Jul. 25, 1989).
Bol, J.F., et al., "Plant Pathogenesis–Related Proteins Induced by Virus Infection", *Annu. Rev. Phytopathol.,* 28, 113–138 (1990).
Depicker, A.G., et al., "A Negative Selection Scheme for Tobacco Protoplast–Derived Cells Expressing the T–DNA Gene 2", *Plant Cell Reports,* 7, 63–66 (1988).
Domoney, C., et al., "Cloning and Characterization of Complementary DNA for Convicilin, a Major Seed Storage Protein in *Pisum sativum* L.", *Planta,* 159, 446–453 (1983).
Dunn, G.M., et al., "Inheritance of Cyclic Hydroxamates in *Zea mays* L.", *Can. J. Plant Sci.,* 61, 583–593 (Jul. 1981).
Gepts, P., et al., "Enhanced Available Methionine Concentration Associated with Higher Phaseolin Levels in Common Bean Seeds", *Theor. Appl. Genet.,* 69, 47–53 (1984).
Guerrero, F.D., et al., "Turgor–Responsive Gene Transcription and RNA Levels Increase Rapidly When Pea Shoots are Wilted. Sequence and Expression of Three Inducible Genes", *Plant Mol. Biol.,* 15, 11–26 (1990).
Hu, N.T., et al., "Primary Structure of a Genomic Zein Sequence of Maize", *The EMBO Journal,* 1, 1337–1342 (1982).
Jaworski, J.G., et al., "A Cerulenin Insensitive Short Chain 3–Ketoacyl–Acyl Carrier Protein Synthase in *Spinacia oleracea* Leaves", *Plant Physiol.,* 90, 41–44 (1989).
Josefsson, L.G., et al., "Structure of a Gene Encoding the 1.7 S Storage Protein, Napin, from *Brassica napus", The Journal of Biological Chemistry,* 262, 12196–12201 (Sep. 5, 1987).
Kim, C.S., et al., "Improvement of Nutritional Value and Functional Properties of Soybean Glycinin by Protein Engineering", *Protein Engineering,* 3, 725–731 (1990).
Malan, C., et al., "Correlation Between CuZn Superoxide Dismutase and Glutathione Reductase, and Environmental and Xenobiotic Stress Tolerance in Maize Inbreds", *Plant Science,* 69, 157–166 (1990).
Marks, M.D., et al., "Nucleotide Sequence Analysis of Zein mRNAs from Maize Endosperm", *The Journal of Biological Chemistry,* 260, 16451–16459 (Dec. 25, 1985).
Montoliu, L., et al., "A Tandem of α–Tubulin Genes Preferentially Expressed in Radicular Tissues from *Zea mays", Plant Molecular Biology,* 14, 1–15 (1989).
Mundy, J., et al., "Selective Expression of a Probable Amylase/Protease Inhibitor in Barley Aleurone Cells: Comparison to the Barley Amylase/Subtilisin Inhibitor", *Planta,* 169, 51–63 (1986).
O'Reilly, D.R., et al., "A Baculovirus Blocks Insect Molting by Producing Ecdysteroid UDP–Glucosyl Transferase", *Science,* 245, 1110–1112 (Sep. 8, 1989).
Smith, I.K., et al., "Properties and Functions of Glutathione Reductase in Plants", *Physiol. Plant.,* 77, 449–456 (1989).
Stalker, D.M., et al., "Herbicide Resistance in Transgenic Plants Expressing a Bacterial Detoxification Gene", *Science,* 242, 419–242 (Oct. 21, 1988).
Viotti, A., et al., "Each Zein Gene Class Can Produce Polypeptides of Different Sizes", *The EMBO Journal,* 4, 1103–1110 (1985).
Werr, W., et al., "Structure of the Sucrose Synthase Gene on Chromosome 9 of *Zea mays* L.", *The EMBO Journal,* 4, 1373–1380 (1985).
Yenofsky, R.L., et al., "Isolation and Characterization of a Soybean (*Glycine max*) Lipoxygenase–3 Gene", *Mol. Gen. Genet.,* 211, 215–222 (1988).
Flavell, R., et al., "Prospects for Transforming Monocot Crop Plants", *Nature,* 307, 108–109 (Jan. 12, 1984).
Goodman, R.M., et al., "Gene Transfer in Crop Improvement", *Science,* 236, 48–54 (Apr. 3, 1987).
Paszkowski, J., et al., "Direct Gene Transfer to Plants", *The EMBO Journal,* 3, 2717–2722 (1984).
Potrykus, I., et al., "Direct Gene Transfer to Cells of a Graminaceous Monocot", *Mol. Gen. Genet.,* 199, 183–188 (1985).
Armstrong, C.L., et al., "Genetic and Cytogenetic Variation in Plants Regenerated from Organogenic and Friable, Embryogenic Tissue Cultures in Maize", *Crop Science,* 28, 363–369 (1988).
Dure III, L., et al., "Common Amino Acid Sequence Domains Among the LEA Proteins of Higher Plants", *Plant Molecular Biology,* 12, 475–486 (1989).
Hong, B., et al., "Cloning and Characterization of cDNA Encoding a mRNA Rapidly–Induced by ABA in Barley Aleurone Layers", *Plant Molecular Biology,* 11, 495–506 (1988).
Mundy, J., et al., "Abscisic Acid and Water–Stress Induce the Expression of a Novel Rice Gene", *The EMBO Journal,* 7, 2279–2286 (1988).
Andrews, D.L., et al., "Characterization of the Lipid Acyl Hydrolase Activity of the Major Potato (*Solanum tuberosum*) Tuber Protein, Patatin, by Cloning and Abundant Expression in a Baculovirus Vector", *Biochem. J.,* 252, 199–206 (1988).
Fromm, M.E., et al., "Inheritance and Expression of Chimeric Genes in the Progeny of Transgenic Maize Plants", *Bio/Technology,* 8, 833–839 (1990).

Jaynes, J.M., et al., "Plant Protein Improvement by Genetic Engineering: Use of Synthetic Genes", *Trends in Biotechnology,* 4, 314–320 (Dec. 1986).

Poehlman, J.M., "Backcross Breeding", In: *Breeding Field Crops, 3rd Edition,* AVI Publishing Company, Inc., Westport, CT, 203–206 (1988).

In Vitro Cellular & Developmental Biology, 21, Program Issue: Thirty–Sixth Annual Meeting of the Tissue Culture Association, New Orleans, LA, 88 p. (Mar. 1985).

In Vitro Cellular & Developmental Biology, 23, Program Issue: Thirty–Eighth Annual Meeting of the Tissue Culture Association, Washington, D.C., 93 p. (Mar. 1987).

In Vitro Cellular & Developmental Biology, 24, Program Issue: Thirty–Ninth Annual Meeting of the Tissue Culture Association, Las Vegas, NV, 92 p. (Mar. 1988).

In Vitro Cellular & Developmental Biology, 25, Program Issue: Fortieth Annual Meeting of the Tissue Culture Association, Orlando, FL, 73 p. (Mar. 1989).

"European Firm Devises Insect–Resistant Plants", *Agricultural Biotechnology News,* 1, 6 (Mar.–Apr. 1986).

"Molecular Strategies for Crop Improvement", *Journal of Cellular Biochemistry,* Supplement 14e, List of Plenary and Poster Sessions, organized by Arntzen, C., et al., for The Keystone Conference on Molecular Strategies for Crop Plant Improvement, held at the 19th UCLA Symposia, 257 (1990).

Abbe, E.C., et al., "The Growth of the Shoot Apex in Maize: Embryogeny", *American Journal of Botany,* 41, 285–293 (Apr. 1954).

Adang, M.J., et al., "Expression of a *Bacillus thuringiensis* Insecticidal Crystal Protein Gene in Tobacco Plants", *Molecular Strategies for Crop Protection,* Arntzen, C.J., et al. (eds.), Alan R. Liss, Inc., New York, 345–353 (1987).

Anderson, P.C., et al., "Herbicide–Tolerant Mutants of Corn", *Genome,* 31, 994–999 (1989).

Angus, T.A., "Implications of Some Recent Studies of *Bacillus thuringiensis*—A Personal Purview", *Proceedings of the 4th International Colloquium on Insect Pathology,* College Park, MD, 183–189 (Aug. 25–28, 1970).

Armaleo, D., et al. "Biolistic Nuclear Transformation of *Saccharomyces cerevisiae* and Other Fungi", *Curr. Genet.,* 17, 97–103 (1990).

Aronson, A.I., et al., "*Bacillus thuringiensis* and Related Insect Pathogens", *Microbiological Reviews,* 50, 1–24 (Mar. 1986).

Aronson, J.N., et al., "Toxic Trypsin Digest Fragment from the *Bacillus thuringiensis* Parasporal Protein", *Applied and Environmental Microbiology,* 53, 416–421 (Feb. 1987).

Barton, K.A., et al., "*Bacillus thuringiensis* δ–Endotoxin Expressed in Transgenic *Nicotiana tabacum* Provides Resistance to Lepidopteran Insects", *Plant Physiol.,* 85, 1103–1109 (1987).

Birk, Y., et al., "Separation of a Tribolium–Protease Inhibitor from Soybeans on a Calcium Phosphate Column", *Biochem. Biophys. Acta,* 67, 326–328 (Feb. 12, 1963).

Bishop, D.H., et al., "Genetically Engineered Viral Insecticides—A Progress Report 1986–1989", *Pestic. Sci.,* 27, 173–189 (1989).

Boynton, J.E., et al., "Chloroplast Transformation in Chlamydomonas with High Velocity Microprojectiles", *Science,* 240, 1534–1537 (Jun. 10, 1988).

Bryant, J.A., "At Last: Transgenic Cereal Plants from Genetically Engineered Protoplasts", *Trends in Biotechnology,* 6, 291–292 (Dec. 1988).

Burgerjon, A., et al., "Industrial and International Standardization of Microbial Pesticides—I. *Bacillus thuringiensis", Entomophaga,* 22, 121–129 (1977).

Busvine, J.R., *A Critical Review of the Techniques for Testing Insecticides,* Table of Contents, Commonwealth Agricultural Bureaux, Slough, England, iii–xi (1971).

Bytebier, B., et al., "T–DNA Organization in Tumor Cultures and Transgenic Plants of the Monocotyledon *Asparagus officinalis", Proc. Natl. Acad. Sci. USA,* 84, 5345–5349 (Aug. 1987).

Calabrese, D.M., et al., "A Comparison of Protein Crystal Subunit Sizes in *Bacillus thuringiensis", Canadian Journal of Microbiology,* 26, 1006–1010 (Aug. 1980).

Caplan, A., et al., "Introduction of Genetic Material into Plant Cells", *Science,* 222, 815–821 (Nov. 18, 1983).

Chaleff, R.S., "Induction, Maintenance, and Differentiation of Rice Callus Cultures on Ammonium as Sole Nitrogen Source", *Plant Cell Tissue Organ Culture,* 2, 29–37 (1983).

Christou, P., et al., "Inheritance and Expression of Foreign Genes in Transgenic Soybean Plants", *Proc. Natl. Acad. Sci. USA,* 86, 7500–7504 (Oct. 1989).

Cooksey, K.E., "Purification of a Protein from *Bacillus thuringiensis* Toxic to Larvae of Lepidoptera", *Biochem. J.,* 106, 445–454 (1968).

De Block, M., et al., "Expression of Foreign Genes in Regenerated Plants and Their Progeny", *EMBO J.,* 3, 1681–1689 (1984).

De Block, M., et al., "The Use of Phosphinothricin Resistance as a Selectable Marker in Tobacco Protoplast Transformation", In: *Progress in Plant Protoplast Research,* Proceedings of the 7th International Protoplast Symposium, Wageningen, The Netherlands, Puite, K.J., et al., (eds.), Kluwer Academic Publishers, Dordrecht, 389–390 (Dec. 6–11, 1987).

Denecke, J., et al., "Quantification of Transient Expression Levels of Genes Transferred to Plant Protoplasts by Electroporation", *Progress in Plant Protoplast Research,* Puite, K.J., et al., (eds.), Proceedings of the 7th International Protoplast Symposium, Wageningen, The Netherlands, 337–338 (Dec. 6–11, 1987).

Duncan, D.R., et al., "The Production of Callus Capable of Plant Regeneration for Immature Embryos of Numerous *Zea Mays* Genotypes", *Planta,* 165, 322–332 (1985).

Dunleavy, J.M., "*Curtobacterium plantarum* sp. nov. Is Ubiquitous in Plant Leaves and Is Seed Transmitted in Soybean and Corn", *International Journal of Systematic Bacteriology,* 39, 240–249 (Jul. 1989).

Dybvig, K., et al., "Transposition of Gram–Positive Transposon Tn916 in *Acholeplasma laidlawii* and *Mycoplasma pulmonis", Science,* 235, 1392–1394 (Mar. 13, 1987).

Edallo, S., et al., "Chromosomal Variation and Frequency of Spontaneous Mutation Associated with in vitro Culture and Plant Regeneration in Maize", *Maydica,* 26, 39–56 (1981).

Fast, P.G., et al., "*Bacillus thuringiensis* δ–Endotoxin: Evidence that Toxin Acts at the Surface of Susceptible Cells", *Experientia,* 34, 762–763 (1978).

Faust, R.M., et al., "Bacteria and Their Toxins as Insecticides", In: *Microbial and Viral Pesticides,* Kurstak, E., (ed.), Marcel Dekker, Inc., New York, 75–208 (1982).

Finkle, B.J., et al., "Growth and Regeneration of Alfalfa Callus Lines After Freezing in Liquid Nitrogen", *Plant Science,* 42, 133–140 (1985).

Finney, D.J., In: *Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve,* iii–ix (1952).

Fischhoff, D.A., et al., "Insect Tolerant Transgenic Tomato Plants", *Bio/technology,* 5, 807–812 (1987).

Fukuto, T.R., "Physicochemical Aspects of Insecticidal Action", In: *Insecticidal Biochemistry and Physiology,* Wilkinson, C.F., (ed.), Plenum Press, New York, 397–428 (1976).

Gallagher, S., "Progress and Promise of the Particle Gun", *Ag Biotechnology News,* 6, 12–13 (Mar.–Apr. 1989).

Gallie, D.R., et al., "The 5'–leader Sequence of Tobacco Mosaic Virus RNA Enhances the Expression of Foreign Gene Transcripts in Vitro and in Vivo", *Nucleic Acids Research,* 15, 3257–3273 (1987).

Gatehouse, A.M.R., et al., "Assessment of the Antimetabolic Effects of Trypsin Inhibitors from Cowpea (*Vigna unguiculata*) and Other Legumes on Development of the Bruchid Beetle *Callosobruchus maculatus", J. Sci. Food Agric.,* 34, 345–350 (1983).

Genovesi, A.D., et al., "Embryogenesis in Callus Derived from Rice Microspores", *Plant Cell Reports,* 1, 257–260 (1982).

Georghiou, G.P., et al., "Factors Influencing the Evolution of Resistance", In: *Pesticide Resistance: Strategies and Tactics for Management,* Committee on Strategies for the Management of Pesticide Resistant Pest Populations, Board on Agriculture, National Research Council, National Academy Press, Washington, D.C., 157–169 (1986).

Gerlach, W.L., "Genetic Engineering: Its Place in Plant Breeding", In: *Plant Breeding and Genetic Engineering,* Zakri, A.H., (ed.), Society for the Advancement of Breeding Researches in Asia and Oceania, Bangi, Malaysia, 269–277 (1988).

Goldfarb, B., et al., "Transient Expression of Microprojectile–Introduced DNA in Douglas–Fir", *J. Cell. Biochem.,* 13D, Abstract No. M121, p. 259 (1989).

Goldman, S.L., et al., "Transformation of *Zea mays* by *Agrobacterium tumefaciens:* Evidence for Stable Genetic Alterations", *Journal of Cellular Biochemistry,* 11B, Abstract No. F 202, p. 26 (1987).

Gordon, P.N., et al., "Plant Regeneration from Tissue Cultures of Maize", *Maize Genetics Cooperation Newsletter,* 51, 79–80 (Mar. 1, 1977).

Green, C.E., "New Development in Plant Tissue Culture and Plant Regeneration", In: *Basic Biology of New Developments in Biotechnology,* Hollaender, A., et al., (eds.), Plenum Press, New York, 195–209 (1983).

Green, C.E., "Somatic Embryogenesis and Plant Regeneration from the Friable Callus of *Zea mays", Proceedings of the 5th International Congress on Plant Tissue & Cell Culture,* Tokyo, Japan, 107–108 (1982).

Haccius, B., "Question of Unicellular Origin of Non–Zygotic Embryos in Callus Cultures", *Phytomorphology,* 28, 74–81 (1978).

Harms, C.T., et al., "Regeneration of Plantlets from Callus Cultures of *Zea mays* L.", *Z. Ptlanzenzuchtg. 77,* 347–351 (1976).

Hartree, E.F., "Determination of Protein: A Modification of the Lowry Method that Gives a Linear Photometric Response", *Analytical Biochemistry,* 48, 422–427 (1972).

Harvey, W.R., et al., "Potassium Ion Transport ATPase in Insect Epithelia", *J. Exp. Biol.,* 106, 91–117 (1983).

Heimpel, A.M., et al., "Recent Advances in the Knowledge of Some Bacterial Pathogens of Insects", *Proceedings of the Tenth International Congress of Entomology,* vol. 4, 711–722 (1956).

Heimpel, A.M., et al., "The Site of Action of Crystalliferous Bacteria in Lepidoptera Larvae", *Journal of Insect Pathology,* 1, 152–170 (1959).

Hernalsteens, J.–P., et al., "An Agrobacterium–Transformed Cell Culture from the Monocot *Asparagus Officinalis", The EMBO Journal,* 3, 3039–3041 (Dec. 1984).

Hibberd, K.A., "Induction, Selection, and Characterization of Mutants in Maize Cell Cultures", In: *Cell Culture and Somatic Cell Genetics of Plants,* vol. 1, Vasil, I.K., (ed.), Academic Press, Inc., Orlando, FL, 571–576 (1984).

Hickle, L.A., et al., "Analytical Chemistry of *Bacillus thuringiensis:* An Overview", In: *Analytical Chemistry of Bacillus thuringiensis,* Hickle, L.A., et al., (eds.), Developed from a Symposium Sponsored by the Division of Agrochemicals at the 198th National Meeting of the American Chemical Society, Miami Beach, FL, vii–ix, 1–8 (Sep. 10–15, 1989).

Hilder, V.A., et al., "A Novel Mechanism of Insect Resistance Engineered into Tobacco", *Nature,* 330, 160–163 (Nov. 12, 1987).

Hodges, T.K., et al., "Genotype Specificity of Somatic Embryogenesis and Regeneration in Maize", *Bio/technology,* 4, 219–223 (Mar. 1986).

Hodges, T.K., et al., "Regeneration of Maize", In: *Biotechnology in Plant Science,* Zaitlin, M., et al., (ed.), Academic Press, Inc., Orlando, FL, 15–33 (1985).

Hoekema, A., et al., "Codon Replacement in the PGK1 Gene of *Saccharomyces cerevisiae:* Experimental Approach to Study the Role of Biased Codon Usage in Gene Expression", *Molecular and Cellular Biology,* 7, 2914–2924 (Aug. 1987).

Hofmann, C., et al., "Binding of the Delta Endotoxin from *Bacillus thuringiensis* to Brush–Border Membrane Vesicles of the Cabbage Butterfly (*Pieris Brassicae*)", *Eur. J. Biochem.,* 173, 85–91 (1988).

Hofmann, C., et al., "Specificity of *Bacillus thuringiensis* δ–Endotoxins is Correlated with the Presence of High–Affinity Binding Sites in the Brush Border Membrane of Target Insect Midguts", *Proc. Natl. Acad. Sci. USA,* 85, 7844–7848 (Nov. 1988).

Höfte, H., et al., "Monoclonal Antibody Analysis and Insecticidal Spectrum of Three Types of Lepidopteran–Specific Insecticidal Crystal Proteins of *Bacillus thuringiensis", Applied and Environmental Microbiology,* 54, 2010–2017 (Aug. 1988).

Höfte, H., et al., "Structual and Functional Analysis of a Cloned Delta Endotoxin of *Bacillus thuringiensis berliner* 1715", *Eur. J. Biochem.,* 161, 273–280 (1986).

Hollingworth, R.M., "The Biochemical and Physiological Basis of Selective Toxicity", In: *Insecticidal Biochemistry and Physiology,* Wilkinson, C.F., (ed.), Plenum Press, New York, 431–506 (1976).

Horsch, R.B., et al., "A Simple and General Method for Transferring Genes into Plants", *Science,* 227, 1229–1231 (Mar. 8, 1985).

Huber, H.E., et al., "*Bacillus thuringiensis* δ–Endotoxin: Composition and Activation", In: *Pathogenesis of Invertebrate Microbial Diseases,* Davidson, E.W., (ed.), Allanheld, Osmun & Co. Publishers, Inc., Totowa, NJ, 209–234 (1981).

Huber–Lukac, M., et al., "Characterization of Monoclonal Antibodies to a Crystal Protein of *Bacillus thuringiensis* subsp. *kurstaki", Infection and Immunity,* 54, 228–232 (Oct. 1986).

Imbrie–Milligan, C.W., et al., "Microcallus Formation from Maize Protoplasts Prepared from Embryogenic Callus", *Planta,* 168, 395–401 (1986).

Jarrett, P., "Potency Factors in the delta–Endotoxin of *Bacillus thuringiensis* var. *aizawi* and the Significance of Plasmids in their Control", *Journal of Applied Bacteriology,* 58, 437–448 (1985).

Johnson, D.E., "Toxicity of *Bacillus thuringiensis* Entomocidal Protein Toward Cultured Insect Tissue", *Journal of Invertebrate Pathology,* 38, 94–101 (1981).

King, P., et al., "Maize", In: *Handbook of Plant Cell Culture,* vol. 2, Sharp, W.R., et al., (eds.), Macmillan Publishing Company, New York, 69–91 (1984).

Klein, T.M., et al., "Advances in Direct Gene Transfer into Cereals", In: *Genetic Engineering: Principles and Methods,* vol. 11, Setlow, J.K., (ed.), Plenum Publishing Corp., New York, 13–31 (1989).

Klein, T.M., et al., "Particle Gun Technology: A Novel Method for the Introduction of DNA into Living Cells", *Program and Abstracts for an International Symposium: "Biotechnology in Plant Science: Relevance to Agriculture in the Eighties",* Poster, #28, Ithaca, NY, 25 (Jun. 23–27, 1985).

Klein, T.M., et al., "Stable Genetic Transformation of Intact Nicotiana Cells by the Particle Bombardment Process", *Proc. Natl. Acad. Sci. USA,* 95, 5502–5505 (Nov. 1988).

Knowles, B.H., et al., "Characterization and Partial Purification of a Plasma Membrane Receptor for *Bacillus thuringiensis* var. *Kurstaki* Lepidopteran–Specific δ–Endotoxin", *J. Cell Sci.,* 83, 89–101 (1986).

Knowles, B.H., et al., "Lectin–Like Binding of *Bacillus thuringiensis* var. *Kurstaki* Lepidopteran–Specific Toxin is an Initial Step in Insecticidal Action", *FEBS Letters,* 168, 197–202 (Mar. 1984).

Langridge, W.H., et al., "Electric Field Mediated DNA Transformation in Plant Protoplasts", *Program and Abstracts for an International Symposium: "Biotechnology in Plant Science: Relevance to Agriculture in the Eighties",* Ithaca, NY, Poster #30, p. 25 (Jun. 23–27, 1985).

Leason, M., et al., "Inhibition of Pea Leaf Glutamine Synthetase by Methionine Sulphoximine, Phosphinothricin and Other Glutamate Analogues", *Biochemistry,* 21, 855–857 (1982).

Lee, B., "Cereal Transformation", *Plant Today,* 9–11 (Jan–Feb. 1989).

Lörz, H., et al., "Gene Transfer to Cereal Cells Mediated by Protoplast Transformation", *Mol. Gen. Genet.,* 199, 178–182 (1985).

Lowe, K., et al., "Plant Regeneration via Organogenesis and Embryogenesis in the Maize Inbred Line B73", *Plant Science,* 41, 125–132 (1985).

Luckow, V.A., et al., "Trends in the Development of Baculovirus Expression Vectors", *Bio/Technology,* 6, 47–55 (Jan. 1988).

Lüthy, P., "Insecticidal Toxins of *Bacillus thuringiensis", FEMS Microbiology Letters,* 8, 1–7 (1980).

Mangano, M.L., et al., "Long–Term Cold Storage of Regenerable Maize Callus", *In Vitro Cellular and Developmental Biology,* 25, Abstract No. 224, p. 66A (Mar. 1989).

Merryweather, A.T., et al., "Construction of Genetically Engineered Baculovirus Insecticides Containing the *Bacillus thuringiensis* subsp. *Kurstaki* HD–73 Delta Endotoxin", *Journal of General Virology,* 71, 1535–1544 (1990).

Molnar, S.J., et al., "Initiation of Totipotent Tissue Cultures from Undeveloped Axillary and Secondary Ears", *Maize Genetics Cooperation Newsletter,* 54, 52–53 (Mar. 31, 1980).

Murphy, D.W., et al., "*Bacillus thuringiensis* Enzyme–Digested Delta Endotoxin: Effect on Cultured Insect Cells", *Science,* 194, 954–956 (Nov. 26, 1976).

Nishiitsutsuji–Uwo, J., et al., "Mode of Action of *Bacillus thuringiensis* δ–Endotoxin: Effect on TN–368 Cells", *Journal of Invertebrate Pathology,* 34, 267–275 (1979).

Ochatt, S.J., et al., "Selection for Salt/Drought Tolerance using Isolated Protoplasts and Protoplast–Derived Calli of Colt Cherry (*Prunus avium x pseudocerasus*)", In: *Progress in Plant Protoplast Research,* Puite, K.J., et al., (eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 391–392 (1988).

Oeda, K., et al., "Formation of Crystals of the Insecticidal Proteins of *Bacillus thuringiensis* subsp. *aizawai* IPL7 in *Escherichia coli", Journal of Bacteriology,* 171, 3568–3571 (Jun. 1989).

Park, W.D., et al., "High–Level, Sucrose–Inducible Expression of a Chimeric Patatin–GUS Gene In Leaf Explants of Transgenic Tobacco Plants", *Journal of Cellular Biochemistry,* 13D, Abstract No. M 343, p. 310 (Mar. 27–Apr. 7, 1989).

Perlak, F.J., et al., "Expression of *Bacillus thuringiensis* Proteins in Transgenic Plants", In: *Biotechnology, Biological Pesticides and Novel Plant–Pest Resistance for Insect Pest Management,* Roberts, D.W., et al., (eds.), Insect Pathology Resource Center, Boyce Thompson Institute for Plant Research, Cornel University, Ithaca, NY, 77–81 (1988).

Poehlman, J.M., et al., In: *Breeding Field Crops,* 3rd Edition, AVI Publishing Company, Inc., Westport, CT, 149–152 (1987).

Poethig, R.S., "Maize—The Plant and Its Parts", In: *Maize for Biological Research,* Sheridan, W.F., (ed.), Plant Molecular Biology Association., Charlottesville, VA, 9–18 (1982).

Potrykus, I., et al., "Direct Gene Transfer: State of the Art and Future Potential", *Plant Molecular Biology Reporter,* 3, 117–128 (Summer 1985).

Randolph, L.F., et al., "Developmental Morphology of the Caryopsis in Maize", *Journal of Agricultural Research,* 53, 881–916 (Dec. 15, 1936).

Rhodes, C.A., et al., "Cytogenetic Stability of Aneuploid Maize Tissue Cultures", *Can. J. Genet. Cytol.,* 28, 374–384 (1986).

Rhodes, C.A., et al., "Factors Affecting Tissue Culture Initiation from Maize Tassels", *Plant Science,* 46, 225–232 (1986).

Rice, T.B., "Tissue Culture Induced Genetic Variation in Regenerated Maize Inbreds", *Proceedings of the 37th Annual Corn & Sorghum Industry Research Conference,* 148–162 (1982).

Rosahl, S., et al., "Expression of a Tuber–Specific Storage Protein In Transgenic Tobacco Plants: Demonstration Of An Esterase Activity", *EMBO. J,* 6, Press Limited, Oxford, England, 1155 (1987).

Roth, B.A., et al., "Genetic Regulation of Transient Expression of Maize Anthocyanin Pathway Genes Introduced into Intact Maize Tissues by Microprojectile Bombardment", *Journal of Cellular Biochemistry,* 13D, Abstract No. M 344, p. 310 (Mar. 27–Apr. 7, 1989).

Roush, R.T., et al., "Ecological Genetics of Insecticidal and Acaricide Resistance", *Ann. Rev. Entomol.,* 32, 361–380 (1987).

Ryan, A.J., et al., "The Expression of the Napin Gene Under the Control of Its Own Promoter in Transgenic Tobacco Plants", *Journal of Cellular Biochemistry,* 13D, Abstract No. M 345, p. 310 (Mar. 27–Apr. 7, 1989).

Sanford, J.C., "The Biolistic Process", *Plant Physiology,* 89, Abstract No. 9, p. 2 (Apr. 1989).

Sanford, J.C., "Delivery of DNA into Regenerable Tissues of Monocots, Using High–Velocity Microprojectiles", Grant Application No. 86–0183, United States Department of Agriculture, Science and Education, 57 p. (Feb. 27, 1986).

Sass, J.E., "Comparative Leaf Number in the Embryos of Some Types of Maize", *Iowa State Coll. J. Sci.,* 25, 509–512 (1951).

Schafer, W., et al., "T–DNA Integration and Expression in a Monocot Crop Plant after Induction of Agrobacterium", *Nature,* 327, 529–532 (Jun. 11, 1987).

Schardl, C.L., et al., "Design and Construction of a Versatile System for the Expression of Foreign Genes in Plants", *Gene,* 61, 1–11 (1987).

Schnepf, H.E., et al., "Delineation of a Toxin–Encoding Segment of a *Bacillus thuringiensis* Crystal Protein Gene", *The Journal of Biological Chemistry,* 260, 6273–6280 (1985).

Shaner, D.L., et al., "Mechanism of Action of the Imidazolinones and Cell Culture Selection of Tolerant Maize", In: *Biotechnology in Plant Sciences,* Zaitlin, M., et al., (eds.), Academic Press, Orlando, FL, 287–299 (1985).

Sharman, B.C., "Developmental Anatomy of the Shoot of *Zea mays* L.", *Annals of Botany, VI,* 246–281 (Apr. 1942).

Shields, R., "Towards Insect–Resistant Plants", *Nature,* 328, 12–13 (Jul. 2, 1987).

Shivakumar, A.G., et al., "Vegetative Expression of the δ–Endotoxin Genes of *Bacillus thuringiensis* subsp. *kurstaki* in *Bacillus subtilis", Journal of Bacteriology,* 166, 194–204 (Apr. 1986).

Smith, G.E., et al., "Molecular Engineering of the *Autographa californica* Nuclear Polyhedrosis Virus Genome: Deletion Mutations Within the Polyhedrin Gene", *Journal of Virology,* 46, 584–593 (May 1983).

St. Julian, G., et al., "Bacteria, Spirochetes, and Rickettsia as Insecticides", *Annals of the New York Academy of Sciences,* 217, 65–75 (1973).

Stolle, C.A., et al., "Cellular Factor Affecting the Stability of β–globulin mRNA", *Gene,* 62, 65–74 (1988).

Strauch, E., et al., "Cloning of a Phosphinothricin N–Acetyltransferase Gene from Streptomyces Viridochromogenes Tu494 and its Expression in *Streptomyces lividans* and *Escherichia coli", Gene,* 63, 65–74 (1988).

Stroo, H.F., et al., "Heterotrophic Nitrification in an Acid Forest Soil and by an Acid–Tolerant Fungus", *Applied and Environmental Microbiology* , 52, 1107–1111 (Nov. 1986).

Suprasanna, P., et al., "Plantlet Regeneration from Glume Calli of Maize (*Zea mays* L.)", *Theor. Appl. Genet.,* 72, 120–122 (1986).

Thomas, W.E., et al., "Mechanism of Action of *Bacillus thuringiensis* var *israelensis* Insecticidal δ–Endotoxin", *FEBS Letters,* 154, 362–368 (Apr. 1983).

Tojo, A., et al., "Dissolution and Degradation of *Bacillus thuringiensis* δ–Endotoxin by Gut Juice Protease of the Silkworm *Bombyx mori", Applied and Environmental Microbiology,* 45, 576–580 (Feb. 1983).

Tomes, D.T., "Cell Culture, Somatic Embryogenesis and Plant Regeneration in Maize, Rice, Sorghum and Millets", In: *Cereal Tissue and Cell Culture,* Bright, S.W.J., et al., (eds.), Martinus Nijnoff/Dr. W. Junk, Amsterdam, The Netherlands, 175–203 (1985).

Tomes, D.T., "Initiation of Embryogenic Callus Cultures from Immature Embryos of Elite Corn (*Zea mays* L.) Germplasm", *In Vitro,* 20, Abstract No. 146, p. 276 (Mar. 1984).

Tomes, D.T., et al., "The Effect of Parental Genotype on Initiation of Embryogenic Callus from Elite Maize (*Zea mays* L.) Germplasm", *Theor. Appl. Genet.,* 70, 505–509 (1985).

Torne, J.M., et al., "Regeneration of Plants from Mesocotyl Tissue Cultures of Immature Embryos of *Zea mays* L.", *Plant Science Letters,* 17, 339–344 (1980).

Vaeck, M., et al., "*Bacillus thuringiensis* Endotoxin Gene Expression and Insect Resistance in Higher Plants", *Pesticide Science,* 20, 319–320 (1987).

Vaeck, M., et al., "Engineering Improved Crops for Agriculture: Protection from Insects and Resistance to Herbicides", In: *Plant Gene Systems and Their Biology,* Key, J.L., et al., (eds.), Alan R. Liss, Inc., New York, 171–181 (1987).

Vaeck, M., et al., "Engineering of Insect Resistant Plants Using a *B. thuringiensis* Gene", In: *Molecular Strategies for Crop Protection,* New York, Alan R. Liss, Inc., 355–366 (1987).

Vaeck, M., et al., "Insect Resistance in Transgenic Plants Expressing *Bacillus thuringiensis* Toxin Gens", *An. Soc. Entomol. Brasil,* 16, 427–435, (1987).

Vaeck, M., et al., "Protein Engineering in Plants: Expression of *Bacillus thuringiensis* Insecticidal Protein Genes", *Cell Culture and Somatic Cell Genetics of Plants,* 6, 425–439, (1989).

Vaeck, M., et al., "Transgenic Plants Protected from Insect Attack", *Nature,* 328, 33–37, (Jul. 2, 1987).

van den Elzen, P.J., et al., "A Chimaeric Hygromycin Resistance Gene as a Selectable Marker in Plant Cells", *Plant Molecular Biology,* 5, 299–302, (1985).

van den Elzen, P.J., et al., "Simple Binary Vectors for DNA Transfer to Plant Cells", *Plant Molecular Biology,* 5, 149–154, (1985).

Van Lammeren, A.A., "Developmental Morphology and Cytology of the Young Maize Embryo (*Zea mays* L.)", *Acta Bot. Neerl.,* 35, 169–188 (Aug. 1986).

Vasil, I.K., "Isolation and Culture of Protoplasts of Grasses", *International Review of Cytology,* Supplement 16, Bourne, G.H., et al., (eds.), Academic Press, New York, 79–88 (1983).

Vasil, V., et al., "Histology of Somatic Embryogenesis in Cultured Immature Embryos of Maize (*Zea mays* L.)", *Protoplasma,* 127, 1–8 (1985).

Watson, S.A., "Corn Marketing, Processing and Utilization", In: *Corn and Corn Improvement,* 3rd Edition, Sprague, G.F., et al., (eds.), American Society of Agronomy, Inc., et al., Madison, WI, 881–939 (1988).

Weigel, Jr., R.C., et al., "Somatic Embryogenesis in Barley", *In Vitro,* 20, Abstract No. 147, p. 277 (Mar. 1984).

Weissinger, A., et al., "Maize Transformation via Microprojectile Bombardment", In: *Genetic Improvements of Agriculturally Important Crops,* Fraley, R.T., et al., (eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 21–25 (1988).

Weissinger, A., et al., "Microprojectile Bombardment for Maize Transformation", *In Vitro Cellular and Developmental Biology,* 23, Program Issue, 38th Annual Meeting of the Tissue Culture Association, Washington, D.C., Abstract No. 254 (Mar.1987).

Wernicke, W., et al., "Adventitious Embryoid and Root Formation from Rice Leaves", *Z. Pflanzenphysiol. Bd.,* 103, 361–365 (1981).

Withers, L., et al., "Proline: A Novel Cryoprotectant for the Freeze Preservation of Cultured Cells of *Zea mays* L.", *Plant Physiology,* 64, 675–678 (1979).

Witt, D.P., et al., "Cytotoxicity of *Bacillus thuringiensis* δ–Endotoxins to Cultured Cf–1 Cells Does Not Correlate with In Vivo Activity Toward Spruce Budworm Larvae", In: *Fundamental and Applied Aspects of Invertebrate Pathology,* Samson, R.A., et al., (eds.), Fourth International Colloquium of Invertebrate Pathology, Wangingen, The Netherlands, 3–6 (Aug. 18–22, 1986).

Wohlleben, W., et al., "Nucleotide Sequence of the Phosphinothricin N–Acetyltransferase Gene from *Streptomyces viridochromogenes* Tü494 and Its Expression in *Nicotania tabacum", Gene,* 70, 25–37 (1988).

Wood, M., "Blast Those Genes!", *Agricultural Research,* 2 p. (Jun. 1989).

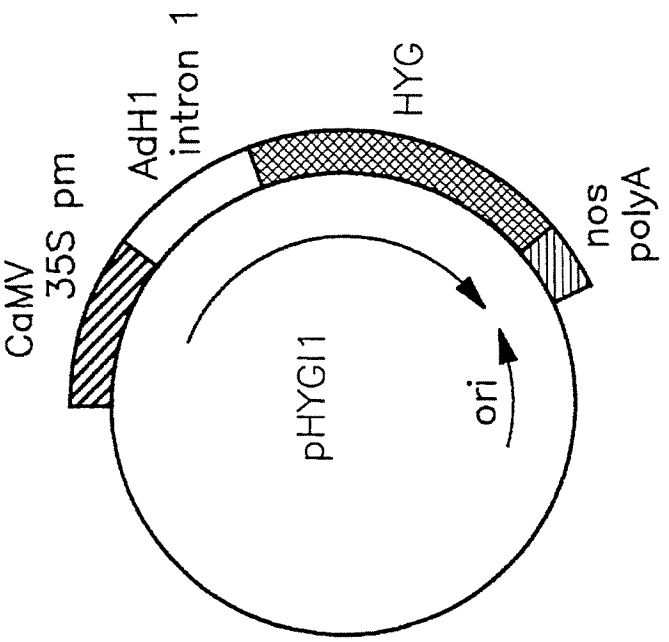

CaMV
35S pm
AdH1
intron 1
HYG
nos
polyA
pHYGI1
ori

FIG. 1A

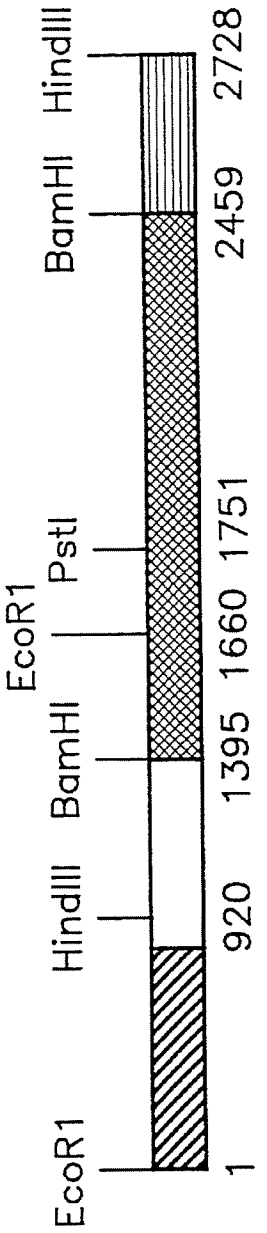

EcoR1
HindIII
BamHI
EcoR1
PstI
BamHI
HindIII
1
920
1395
1660
1751
2459
2728

FIG. 1B

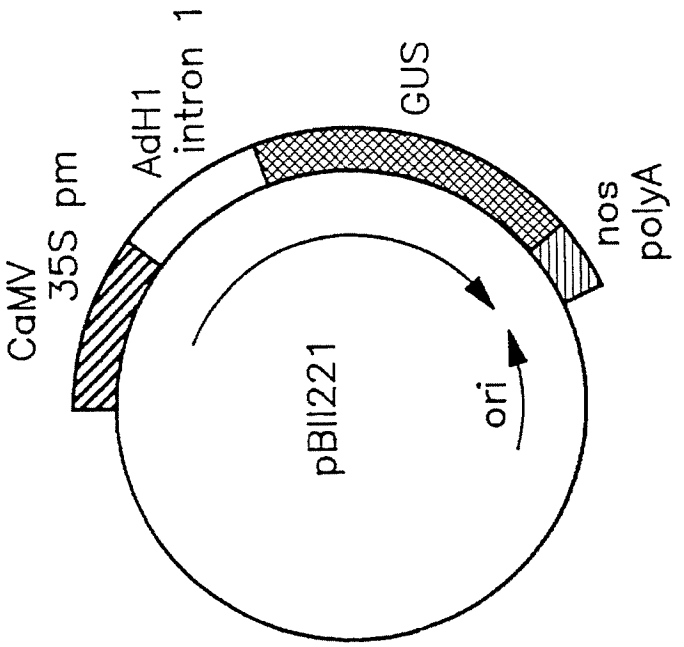
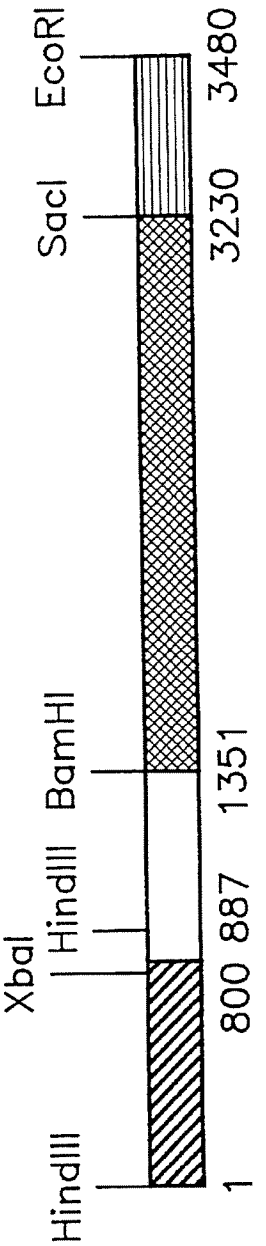

CaMV 35S pm
AdH1 intron 1
GUS
nos polyA
pBII221
ori

FIG. 2A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3480
SacI
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BamHI
1351
XbaI
HindIII
887
800
HindIII
1

FIG. 2B

FIG. 3

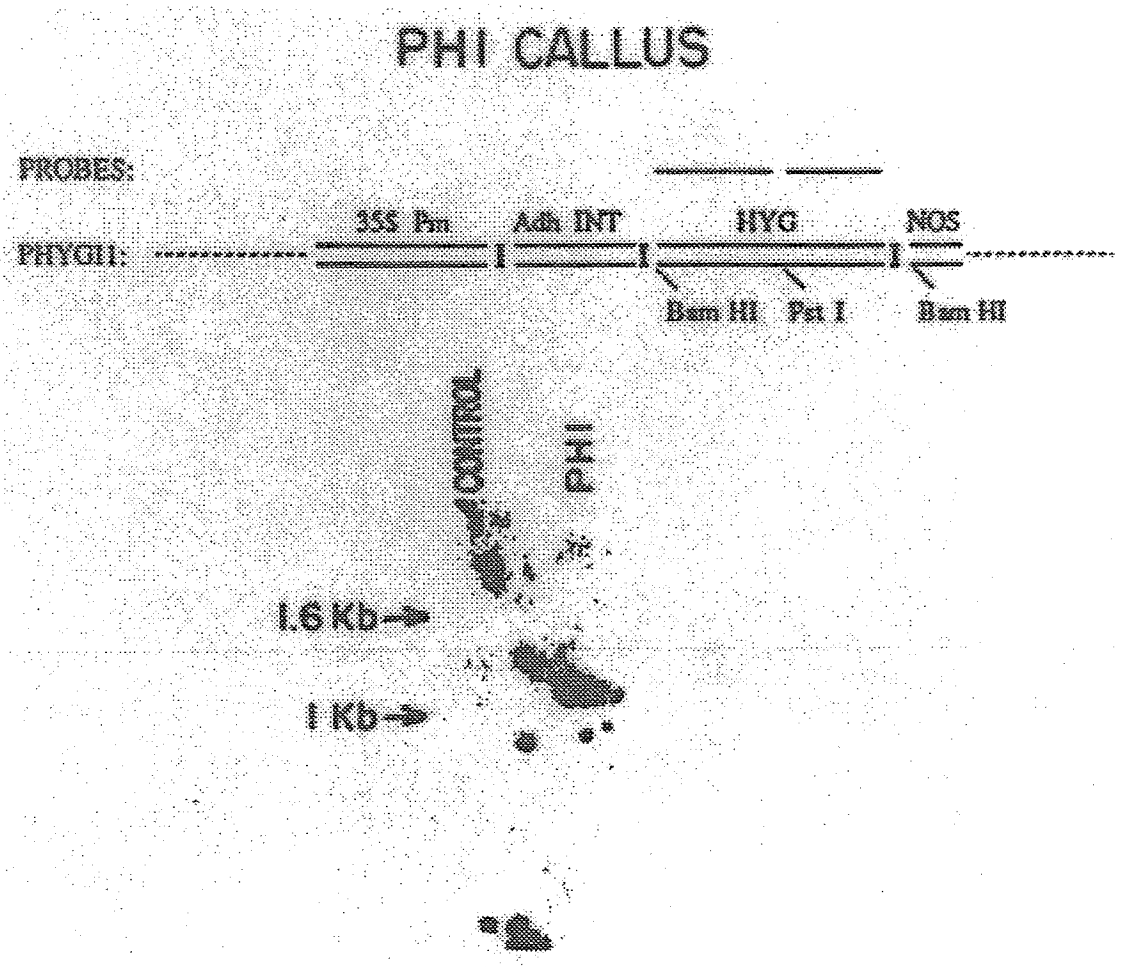
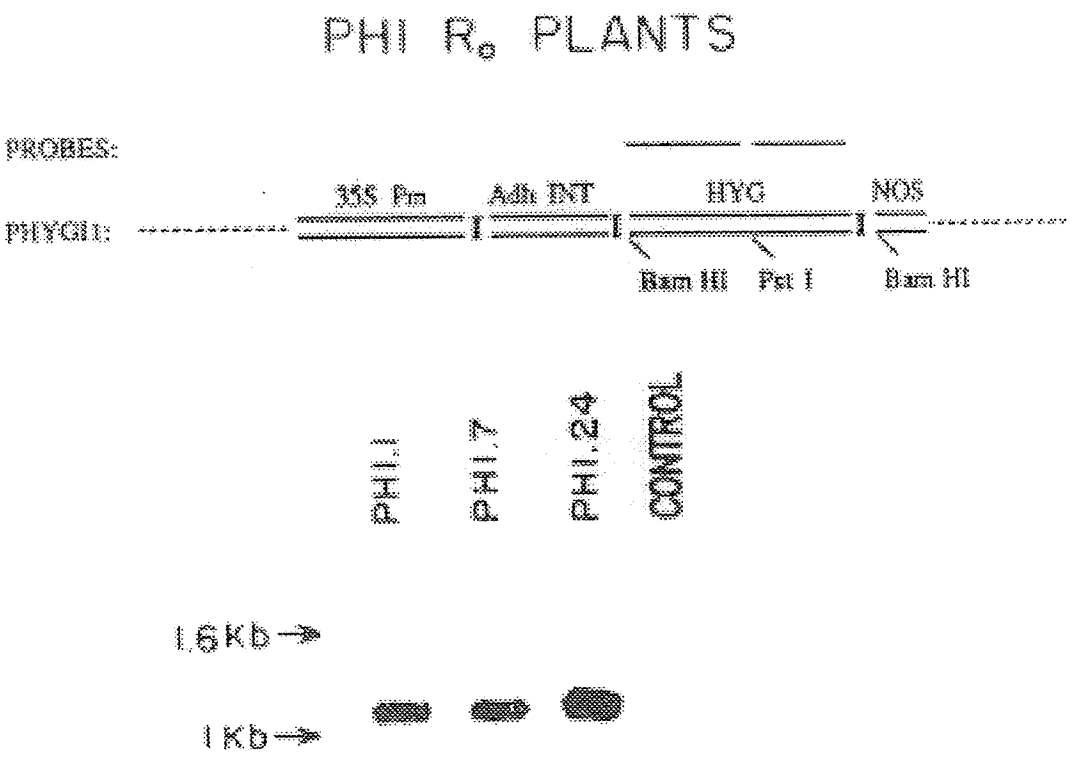

PHI CALLUS
PROBES:
PHYG11:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI
CONTROL
PHI
1.6 Kb
1 Kb

FIG. 4


PHI R_o PLANTS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
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pHI.24
CONTROL
1.6Kb
1Kb

FIG. 5

PHI R_1 GENERATION

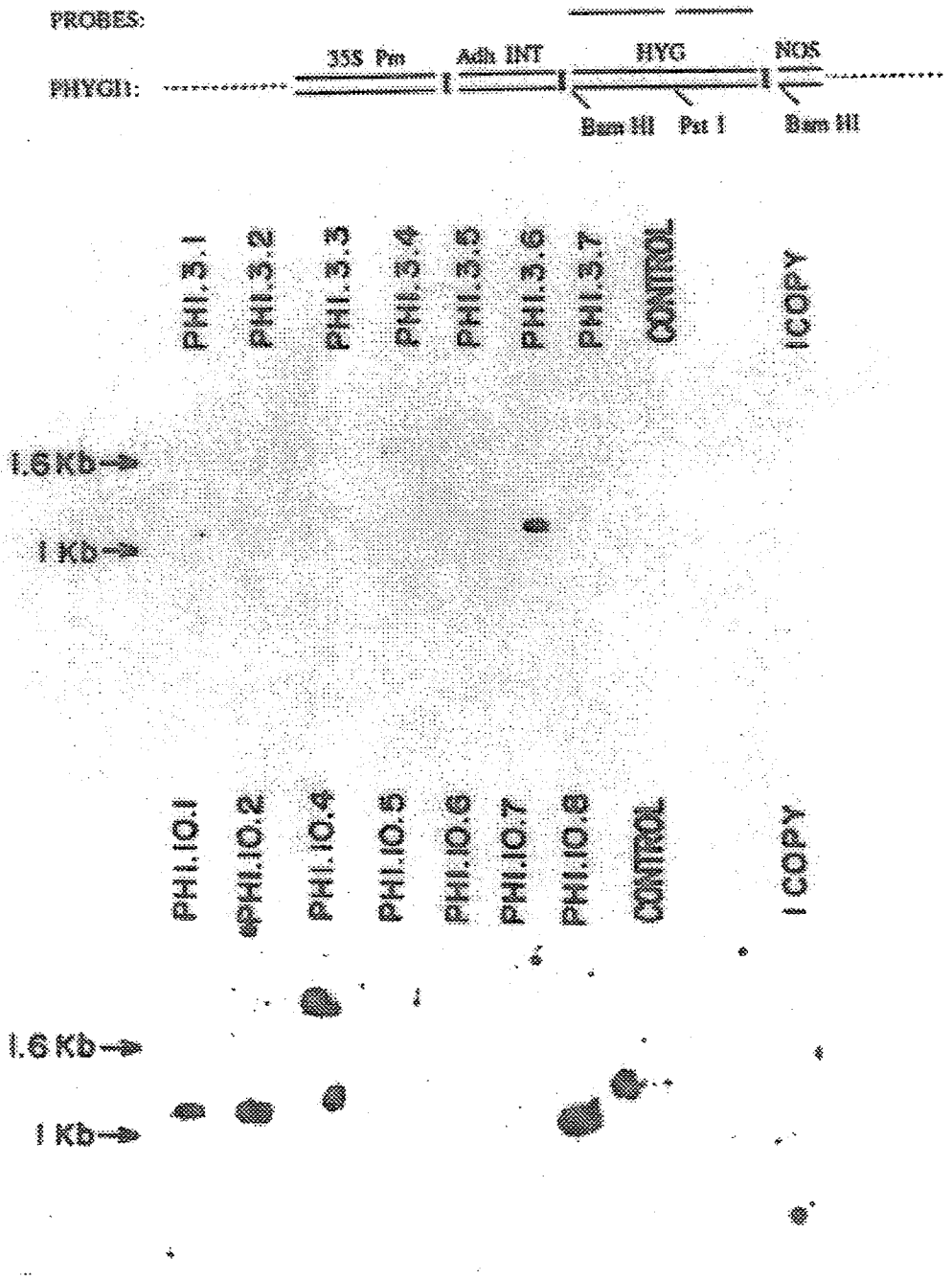
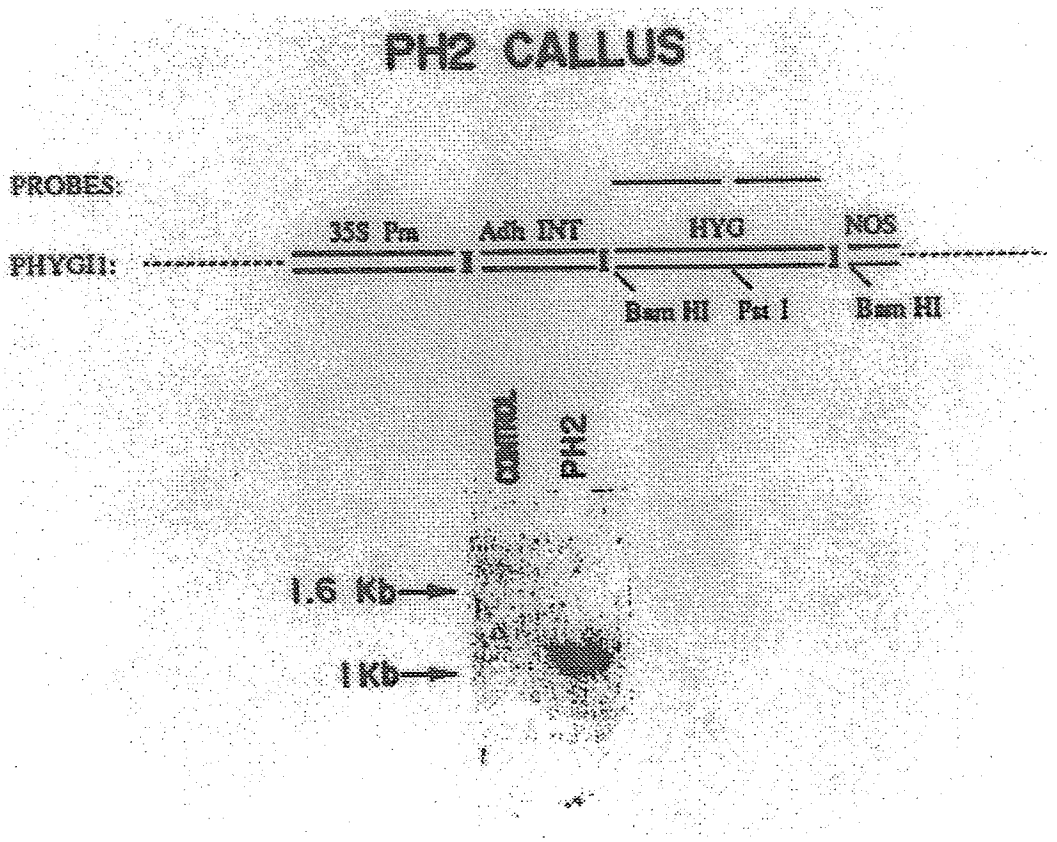


FIG. 6


PH2 CALLUS
PROBES:
PHYGII:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI
CONTROL
PH2
1.6 Kb
1 Kb

FERTILE TRANSGENIC CORN PLANTS

This application is a division of U.S. patent application Ser. No. 08/677,695, filed Jul. 10, 1996, which is a continuation of U.S. patent application Ser. No. 07/974,379, filed Nov. 10, 1992, now U.S. Pat. No. 5,538,877, which in turn is a continuation of U.S. patent application Ser. No. 07/467,983, filed Jan. 22, 1990, now abandoned.

BACKGROUND OF THE INVENTION

This invention relates to fertile transgenic plants of the species *Zea mays* (oftentimes referred to herein as maize or corn). The invention further relates to producing transgenic plants via particle bombardment and subsequent selection techniques which have been found to produce fertile transgenic plants.

Genetic engineering of plants, which entails the isolation and manipulation of genetic material (usually in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells, offers considerable promise to modern agriculture and plant breeding. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals, chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques. Once a gene has been identified, cloned, and engineered, it is still necessary to introduce it into a plant of interest in such a manner that the resulting plant is both fertile and capable of passing the gene on to its progeny.

A variety of methods have been developed and are currently available for the transformation of various plants and plant cells with DNA. Generally these plants have been dicotyledonous, and some success has been reported with certain of the monocotyledonous cereals. However, some species have heretofore proven untransformable by any method. Thus, previous to this discovery, no technology had been developed which would permit the production of stably transformed *Zea mays* plants in which the transforming DNA is heritable thereof. This failure in the art is well documented in the literature and has been discussed in a number of recent reviews (Potrykus, 1989; Weising et al., 1988; Cocking et al., 1987).

European Patent Publns. 270,356 (McCabe et al.) and 275,069 (Arntzen et al.) describe the introduction of DNA into maize pollen followed by pollination of maize ears and formation of seeds. The plants germinated from these seeds are alleged to contain the introduced DNA, but there is no suggestion that the introduced DNA was heritable, as has been accomplished in the present invention. Only if the DNA introduced into the corn is heritable can the corn be used in breeding programs as required for successful commercialization of transgenic corn.

Graves et al. (1986) claims Acrobacterium-mediated transformation of *Zea mays* seedlings. The alleged evidence was based upon assays known to produce incorrect results.

Despite extensive efforts to produce fertile transformed corn plants which transmit the transforming DNA to progeny, there have been no reported successes. Many previous failures have been based upon gene transfer to maize protoplasts, oftentimes derived from callus, liquid suspension culture cells, or other maize cells using a variety of transformation techniques. Although several of the techniques have resulted in successful transformation of corn cells, the resulting cells either could not be regenerated into

corn plants or the corn plants produced were sterile (Rhodes et al. 1988) or, in some cases, it even turned out that the plants were in fact not transformed. Thus, while maize protoplasts and some other cells have previously been transformed, the resulting transformants could not be regenerated into fertile transgenic plants.

On the other hand, it has been known that at least certain corn callus can be regenerated to form mature plants in a rather straightforward fashion and that the resulting plants were often fertile. However, no stable transformation of maize callus was ever achieved, i.e. there were no techniques developed which would permit a successful stable transformation of a regenerable callus. An example of a maize callus transformation technique which has been tried is the use of Agrobacterium mediated transfer.

The art was thus faced with a dilemma. While it was known that corn protoplast and suspension culture cells could be transformed, no techniques were available which would regenerate the transformed protoplast into a fertile plant. While it was known that corn callus could be regenerated into a fertile plant, there were no techniques known which could transform the callus, particularly while not destroying the ability of the callus both to regenerate and to form fertile plants.

Recently, a new transformation technique has been created based upon-the bombardment of intact cells and tissues with DNA-coated microprojectiles. The technique, disclosed in Sanford et al. (1987) as well as in EPO Patent Publication 331,855 of J. C. Sanford et al. based upon U.S. Ser. No. 161,807, filed Feb. 29, 1988, has been shown effective at producing transient gene expression in some plant cells and tissues including those from onion, maize (Klein et al. 1988a), tobacco, rice, wheat, and soybean, and stable expression has been obtained in tobacco and soybeans. In fact, stable expression has been obtained by bombardment of suspension cultures of *Zea mays* Black Mexican Sweet (Klein et al. 1989) which cultures are, however, non-regenerable suspension culture cells, not the callus culture cells used in the process of the present invention.

No protocols have been published describing the introduction of DNA by a bombardment technique into cultures of regenerable maize cells of any type. No stable expression of a gene has been reported by means of bombardment of corn callus followed by regeneration of fertile plants and no regenerable fertile corn has resulted from DNA-coated microprojectile bombardment of the suspension cultures. Thus, the art has failed to produce fertile transformed corn plants heretofore.

A further stumbling block to the successful production of fertile transgenic maize plants has been in selecting those few transformants in such a manner that neither the regeneration capacity nor the fertility of the regenerated transformant are destroyed. Due to the generally low level of transformants produced by a transformation technique, the need for selection of the transformants is self-evident. However, selection generally entails the use of some toxic agent, e.g. herbicide or antibiotic, which can effect either the regenerability or the resultant plant fertility.

It is thus an object of the present invention to produce fertile, stably transgenic, *Zea mays* plants and seeds which transmit the introduced gene to progeny. It is a further object to produce such stably transgenic plants and seeds by a particle bombardment and selection process which results in a high level of viability for a few transformed cells. It is a further object to produce fertile stably transgenic plants of other graminaceous cereals besides maize.

REFERENCES CITED

Armstrong, CL, et al. (1985) J Planta 164:207–214
Callis, J, et al. (1987) Genes & Develop 1:1183–1200
Chilton & Barnes (1983) Nuc Acids Res 11:364–385
Chu, CC, et al. (1975) Sci Sin (Peking) 18:659–668
Cocking, F, et al. (1987) Science 236:1259–1262
DeWet et al. (1985) Proc Natl Sci USA 82:7870–7873
Freeling, JC, et al. (1976) Maydica XXI:97–112
Graves, A, et al. (1986) Plant Mol Biol 7:43–50
Green, C, et al. (1975) Crop Sci 15:417–421
Green, CE, (1982) Plant Tissue Culture, A Fujiwara ed. Maruzen, Tokyo, Japan pp 107–8
Green, C, et al. (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 367–372
Gritz, L, et al. (1983) Gene 25:179–188
Guilley, H, et al. (1982) Cell 30:763–773
Jefferson, R, et al. (1987) EMBO J 6:3901–3907)
Kamo, K, et al. (1985) Bot Gaz 146:327–334
Klein, T, et al. (1989) Plant Physiol 91:440–444
Klein, T, et al. (1988a) Proc Natl Acad Sci USA 85:4305–9
Klein, T, et al. (1988b) Bio/Technology 6:559–563
Lu, C, et al. (1982) Theor Appl Genet 62:109–112
McCabe, D, et al. (1988) Bio/Technology 6:923–926
Murashige, T, et al. (1962) Physiol Plant 15:473–497
Neuffer, M, (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 19–30
Phillips, R, et al. (1988) Corn and Corn Improvement, 3rd ed., Agronomy Soc Amer, pp 345–387
Potrykus, I (1989) Trends in Biotechnology 7:269–273
Rhodes, CA, et al. (1988) Science 240:204–7
Sambrook, J, et al (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press
Sanford, J, et al. (1987) J Part Sci & Techn 5:27–37
Weising, K, et al., (1988) Ann Rev of Genetics 22:421–478
Yanisch-Perron, L, et al. (1985) Gene 33:109–119

SUMMARY OF THE INVENTION

The present invention relates to fertile transgenic *Zea mays* plants containing heterologous DNA, preferably chromosomally integrated heterologous DNA, which is heritable by progeny thereof.

The invention further relates to all products derived from transgenic *Zea mays* plants, plant cells, plant parts, and seeds.

The invention further relates to transgenic *Zea mays* seeds stably containing heterologous DNA and progeny which inherit the heterologous DNA.

The invention further relates to a process for producing fertile transgenic *Zea mays* plants containing heterologous DNA. The process is based upon microprojectile bombardment, selection, and plant regeneration techniques.

The invention further relates to a process for producing fertile transformed plants of graminaceous plants other than *Zea mays* which have not been reliably transformed by traditional methods such as electroporation, Afrobacterium, injection, and previous ballistic techniques.

The invention further relates to regenerated fertile mature maize plants from transformed embryogenic tissue, transgenic seeds produced therefrom, and R1 and subsequent generations.

In preferred embodiments, this invention produces the fertile transgenic plants by means of a DNA-coated microprojectile bombardment of clumps of friable embryogenic callus, followed by a controlled regimen for selection of the transformed callus lines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows a map of plasmid vector pHYGI1 utilized in Example I.

FIG. 1B shows the relevant part of pHYGI1 encompassing the HPT coding sequence and associated regulatory elements. The base pair numbers start from the 5' nucleotide in the recognition sequence for the indicated restriction enzymes, beginning with the EcoRI site at the 5' end of the CaMV 35S promoter.

FIG. **2** shows a map of plasmid vector pBII221 utilized in Example I.

FIG. **3** is a Southern blot of DNA isolated from the PH1 callus line and an untransformed control callus line.

FIG. **4** is a Southern blot of leaf DNA isolated from Ro plants regenerated from PH1 and untransformed callus.

FIG. **5** is a Southern blot of leaf DNA isolated from R1 progeny of PH1 Ro plants and untransformed Ro plants.

FIG. **6** is a Southern blot of DNA isolated from the PH2 callus line and an untransformed control callus line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the production of fertile transgenic plants and seeds of the species *Zea mays* and to the plants, plant tissues, and seeds derived from such transgenic plants, as well as the subsequent progeny and products derived therefrom. The transgenic plants produced herein include all plants of this species, including field corn, popcorn, sweet corn, flint corn and dent corn.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant which contains heterologous DNA that was introduced into plant material by a process of genetic engineering, or which was initially introduced into a plant species by such a process and was subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions.

By "heritable" is meant that the DNA is capable of transmission through a complete sexual cycle of a plant, i.e. passed from one plant through its gametes to its progeny plants in the same manner as occurs in normal corn.

The transgenic plants of this invention may be produced by (i) establishing friable embryogenic callus from the plant to be transformed, (ii) transforming said cell line by a microprojectile bombardment technique, (iii) controllably identifying or selecting transformed cells, and (iv) regenerating fertile transgenic plants from the transformed cells. Some of the plants of this invention may be produced from the transgenic seed produced from the fertile transgenic plants using conventional crossbreeding techniques to develop commercial hybrid seed containing heterologous DNA.

I. Plant Lines and Tissue Cultures

The cells which have been found useful to produce the fertile transgenic maize plants herein are those callus cells which are regenerable, both before and after undergoing a selection regimen as detailed further below. Generally, these cells will be derived from meristematic tissue which contain cells which have not yet terminally differentiated. Such tissue in graminaceous cereals in general and in maize, in

particular, comprise tissues found in juvenile leaf basal regions, immature tassels, immature embryos, and coleoptilar nodes. Preferably, immature embryos are used. Methods of preparing and maintaining callus from such tissue and plant types are well known in the art and details on so doing are available in the literature, c.f. Phillips et al. (1988), the disclosure of which is hereby incorporated by reference.

The specific callus used must be able to regenerate into a fertile plant. The specific regeneration capacity of particular callus is important to the success of the bombardment/selection process used herein because during and following selection, regeneration capacity may decrease significantly. It is therefore important to start with cultures that have as high a degree of regeneration capacity as possible. Callus which is more than about 3 months and up to about 36 months of age has been found to have a sufficiently high level of regenerability and thus is currently preferred. The regenerative capacity of a particular culture may be readily determined by transferring samples thereof to regeneration medium and monitoring the formation of shoots, roots, and plantlets. The relative number of plantlets arising per Petri dish or per gram fresh weight of tissue may be used as a rough quantitative estimate of reqeneration capacity. Generally, a culture which will produce at least one plant per gram of callus tissue will be preferred.

While maize callus cultures can be initiated from a number of different plant tissues, the cultures useful herein are preferably derived from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1–3 mm in length. This length generally occurs about 9–14 days after pollination. Under aseptic conditions, the embryos are placed on conventional solid media with the embryo axis down (scutellum up). Callus tissue appears from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" is meant that the tissue is easily dispersed without causing injury to the cells. Tissue with this morphology is then transferred to fresh media and subcultured on a routine basis about every two weeks.

The callus initiation media is solid because callus cannot be readily initiated in liquid medium. The initiation/maintainence media is typically based on the N6 salts of Chu et al. (1975) as described in Armstrong et al. (1985) or the MS salts of Murashige et al. (1962). The basal medium is supplemented with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth. The cultures are generally maintained in the dark, though low light levels may also be used. The level of synthetic hormone 2,4-D, necessary for maintainence and propagation, should be generally about 0.3 to 3.0 mg/l.

Although successful transformation and regeneration has been accomplished herein with friable embryogenic callus, this is not meant to imply that other transformable regenerable cells, tissue, or organs cannot be employed to produce the fertile transgenic plants of this invention. The only actual requirement for the cells which are transformed is that after transformation they must be capable of regeneration of a plant containing the heterologous DNA following the particular selection or screening procedure actually used.

II. DNA Used for Transformation

The heterologous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of a plasmid and contains coding regions of beneficial heterologous DNA with flanking regulatory sequences which serve to promote the expression of the heterologous DNA present in the resultant corn plant. "Heterologous DNA" is used herein to include all synthetically engineered or biologically derived DNA which is introduced into a plant by man by genetic engineering, including but not limited to, non-plant genes, modified genes, synthetic genes, portions of genes, as well as DNA and genes from maize and other plant species.

The compositions of and methods for constructing heterologous DNA for successful transformations of plants is well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the heterologous DNA useful herein. The specific composition of the DNA is not central to the present invention and the invention is not dependent upon the composition of the specific transforming DNA used. weising et al. (1988), the subject matter of which is incorporated herein by reference, describes suitable DNA components thereof which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, as well as provides suitable references for compositions thereof. Sambrook et al. (1989) provides suitable methods of construction.

Generally the heterologous DNA will be relatively small, i.e. less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the DNA increases.

Suitable heterologous DNA for use herein includes all DNA which will provide for, or enhance, a beneficial feature of the resultant transgenic corn plant. For example, the DNA may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. For example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; bacterial ESPS synthase for resistance to glyphosate herbicide; chitinase or glucan endo-1,3-B-glucosidase for fungicidal properties. Also, the DNA may be introduced to act as a genetic tool to generate mutants and/or assist in the identification, genetic tagging, or isolation of segments of corn DNA. Additional examples may be found in Table 1:

TABLE 1

	Eukaryotic genes transferred to higher plants		
Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
Animals			
Drosophila heat shock gene hsp 70	5' hsp70/nptII/3' ocs	tobacco (plants, tumors)	+ temperature-dependent and organ-specific
Drosophila copia element LTR	5' copia/cat	rice, wheat and sorghum (protoplasts)	(–) transient, but strong

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
firefly luciferase gene	5' CaMV 35S/luciferase cDNA/3' nos	tobacco (plants)	–
	5' CaMV 19S/luciferase cDNA/3' nos	carrot (protoplasts)	(+) transient
	5' deletion series		
Rabbit β-globin gene	genomic	tobacco (tumors)	(−) not expressed
Human α-globin gene	5' nos/α-globin	tobacco (plants)	(+) incorrect transcript processing
Chicken α-actin gene	genomic	tobacco (tumors)	(−) not expressed
Chicken oyalbumin gene	genomic	tobacco (tumors)	(+) incorrect transcript processing
Mouse metallothionein gene (mmt)	5' mmt/cat	tobacco (tumors)	(−) not expressed
Mouse dihydrofolate reductase gene (DHFR)	5' CaMV 35S/DHFR-cDNA/ 3' nos	Petunia (plants)	+ expression confers methotrexate resistance
Human growth hormone gene (hgh)	5' CaMV 35S/hgh	tobacco (plants, tumors)	(+) incorrect transcript processing
	5' nos/hgh/3' nos	tobacco and sunflower (tumors)	(+) transcription, but neither processing nor translation
	5' CaMV 35S/hgh/hgh 3'	tobacco (plants)	(+) incorrect transcript polyadenylation
SV40 early genes	5' SV40/cat	tobacco (tumors)	(−) not expressed
	5' CaMV 35S/cat/3' SV40	tobacco (plants)	(+) incorrect transcript polyadenylation
HSV thymidine kinase gene (tk)	5' HSV/tk/cat	tobacco (tumors)	(−) not expressed
Adenovirus type 5 EIA gene	5' CaMV 35S/EIA/ 3' EIA/3' rbcS	tobacco (plants)	(+) termination within rbcS, EIA polyadenylation site not used
Yeast			
Yeast ADH	genomic	tobacco (plants)	(−) not expressed
Plant virus			
cDNA encoding TMV coat protein (tobacco mosaic virus)	5' CaMV 35S/TMVcDNA/ 3' nos	tobacco (plants)	+ expression confers enhanced resistance to TMV infection
cDNA encoding AMV coat protein (alfalfa mosaic virus)	5' CaMV 19S/AMVcDNA 5' CaMV 35S/AMVcDNA/ 3' nos.	tobacco and tomato (plants)	+ expression confers enhanced resistance to AMV infection
cDNAs encoding A- and B-component of TGMV (tomato golden mosaic virus)	separate transformation with either A- or B-component via agroinfection	Petunia (plants)	+ only A-components in tandem are able to replicate
cDNA encoding CMV (cucumber mosaic virus) satellite RNA	5' CaMV 35S/CMVcDNA/ 3' nos	tobacco (plants)	+ expression confers enhanced resistance to CMV infection
Plants			
Bean phaseolin gene	5' ocs/phaseolin genomic genomic	sunflower (tumors)	+ expressed and processed correctly
		tobacco (plants)	+ development-specific expression in seeds: targeting to protein bodies in endosperm and embryos
	5' phaseolin/phaseolin-cDNA/3' phaseolin	tobacco (tumors)	+ higher expression than using a genomic clone
	5' phaseolin/maize zein/ 3' phaseolin	tobacco (plants)	+ development-specific zein gene expression in tobacco seeds. Zein accumulation
Bean phytohemagglutinin-L gene (PHA-L)	genomic	tobacco (plants)	+ development-specific expression in tobacco seeds
Soybean β-conglycinin gene (α subunit)	genomic	Petunia (plants)	+ development-specific expression in Petunia seeds depending on 5' sequences
	5' deletion series		
	5' CaMV 35S or 19S/ conglycinin/3' nos	Petunia (plants)	+ constitutive expression: 35S> 19S; 20 fold clonal variation
Soybean β-conglycinin gene (β subunit)	genomic	tobacco and Petunia (plants)	+ development-specific expression in seeds
Potato patatin gene	5' patatin/cat/3' nos	potato (plants)	+ organ-specific expression in tubers
	5' ST-LS.1/patatin/3' patatin	tobacco (plants)	+ light-regulated and organ-specific expression depending on the ST-LS.1 promoter. Correct splicing of patatin mRNA.
Maize zein gene	genomic	sunflower (tumors)	(+) transcription, but no detectable protein
	5' phaseolin/zein/3' phaseolin	tobacco (plants)	+ development-specific expression in tobacco seeds
Wheat glutenin genes	5' glutenin/cat/3' nos	tobacco (plants)	+ development-specific expression

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
			in tobacco seeds
Wheat chlorophyll a/b-binding protein (cab) gene	genomic	Petunia and tobacco plants	+ light-regulated and organ-specific expression in leaves
	genomic 5' cab/cat 5' cab/5' CaMV 35S/cat/ 3' rbcS 5' deletion series	tobacco (plants)	+ phytochrome-regulated expression in leaves depending on 5' sequences
Pea cab gene	5' cab/nptII 5' cab/5' nos/nptII	tobacco (plants)	+ light-regulated, organ-and cell-specific expression; depending on enhancer/silencer-like 5, sequences. Involvement of phytochrome. Correlation to the presence of chloroplasts.
Petunia cab gene	5' cab/ocs 5' cab/nos	Petunia and tobacco (plants)	+ clonal variation of expression (200 fold) independent of copy number and homo-/ heterologous host genome
Arabidopsis cab gene	5' cab/cat	tobacco (plants)	+ light-regulated and organ-specific expression
Pea ribulose 1.5-Bisphosphate carboxylase small subunit gene (rbcS) E9	genomic 5' rbcS/cat 5' deletion series	Petunia (tumors)	+ light-regulated expression dependent on 5' sequences
	genomic 5' deletion series	Petunia and tobacco (plants)	+ light-regulated and organ-specific expression dependent on 5' sequences: 25-fold clonal variation
Pea rbcS 3.6	5' rbcS/cat/3' nos 5' deletion series	tobacco (tumors)	+ light-regulated expression dependent on enhancer-like 5' sequences
	5' rbcS/rbcS transit sequence/ nptII	tobacco (tumors and plants)	+ light-regulated expression and targeting of neomycin phosphotransferase into chloroplasts; analysis of signal sequences
	5' rbcS/nptII	tobacco (plants)	+ light-regulated, organ- and cell-specific expression. Involvement of a blue-light receptor
Pea rbcS 3A, 3C	genomic	Petunia (plants)	+ regulation of transcription by phytochrome- and/or blue-light receptor depending on the developmental state
Pea rbcS 3A, E9	5' rbcS/5' CaMV 35S/cat 5' deletion series	Petunia and tobacco (plants)	+ light-regulated and organ-specific expression depending on enhancer- and silencer-like 5' sequences
Soybean rbcS	5' rbcS/nptII/3' ocs	soybean (tumors)	+ light-regulated expression
	5' rbcS/nos	Kalanchoe (tumors)	+ light-regulated expression
	5' rbcS/nptII/3' nos	Petunia (plants)	+ light-regulated expression mediated by phytochrome
Soybean, pea and Petunia rbcS	5' rbcS/nptII/3' nos	tomato (plants)	+ expression stronger than directed by nos-promoter
Nicotiana plumbaginifolia rbcS 8B	5' rbcS/cat	tobacco and Petunia (plants)	+ light-regulated and organ-specific expression; 3-fold clonal variation independent of homo-/ heterologous host genome
Wheat rbcS	genomic 5' CaMV 35S/rbcS/3' rbcS	tobacco (plants)	(+) no expression under the control of wheat promoter; CaMV 35S promoter is necessary
Potato ST-LS.1 gene	genomic; modified by exon tagging	potato and tobacco (plants)	+ light-regulated and organ-specific expression depending on the presence of chloroplasts and 5' sequences. Clonal variation parallels copy number, but is independent of homo-/ heterologous host.
	5' ST-LS.1/patatin/3' patatin	tobacco (plants)	+ light-regulated and organ-specific expression depending on the ST-L.1 promoter sequences
Petroselinum and Antirrhinum chalcone synthase genes (chs)	5' chs(A)/nptII/3' chs(P) 5' deletion series	tobacco (plants)	+ UV-B-light-regulated expression dependent on enhancer-like 5' sequences

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
potato proteinase inhibitor II gene (PI II)	genomic 5' PI II/cat/3' T-DNA gene 6b 5' PI II/cat/3' PI II	tobacco (plants)	+ wound-inducible expression depending on 5' and 3' sequences; systemic spreading by transacting factors
Soybean heat shock gene hs 6871	genomic 5' deletion series	sunflower (tumors) and tobacco (plants)	+ temperature-regulated expression depending on 5' sequences
Soybean heat shock gene Gmhsp 17.SE	genomic 5' deletion series	sunflower (tumors)	+ expression regulated by temperature and presence of cadmium and arsenite depending on 5' sequences
Maize heat shock gene hsp70	genomic	Petunia (plants)	+ temperature-regulated expression
Maize alcohol dehydrogenase I gene (AdhI)	5' CaMV 35S/cat/AdhI intron/3' rbcS	tobacco (plants)	(+) maize AdhI intron is not removed from the transcript
	5' AdhI/cat 5' ocs/5' AdhI/cat 5' CaMV 35S/5' AdhI/cat 5' AdhI deletion series	tobacco (plants) and maize (protoplasts)	(+) anaerobically inducible cat-expression dependent on 5' AdhI sequences only if additional CaMV- or ocs-promoter/enhancer sequences are present.
Maize sucrose synthase gene (ss)	5' ss/nptII/3' ocs	wheat (protoplasts)	(+) transient expression
	5' ss/nptII	maize (protoplasts)	(+) transient expression suspension-culture derived but not in leaf-derived protoplasts
Soybean β-tubulin gene	genomic	tobacco (plants)	+
cowpea trypsin inhibitor gene (CpTI)	5' CaMV 35S/CpTI/3' nos	tobacco (plants)	+ expression enhances resistance to insect pests
Petunia EPSP synthase gene	5' CaMV 35S/EPSP/3' nos	Petunia (plants)	+ 35S-directed EPSP overproduction confers glyphosate tolerance
	5' T7-T-DNA/Petunia EPSP transit sequence/bacterial EPSP (aroA) coding region	tobacco (plants)	+ expression of bacterial EPSP; targeting into chloroplasts; glyphosate tolerance
Soybean leghemoglobin gene lbc3	genomic	tobacco (plants)	n.d.
	5' lbc3/cat/3' lbc3	Lotus corniculatus (plants)	+ development-specific and inducible expression only in nodules and only after infection by Rhizobium; dependence on 5' regulatory sequences
Nicatiana plumbaginifolia "insert 7" enhancer-like sequence	5' insert 7/5' nos/nptII	tobacco (plants)	+ transient protoplast-specific overexpression of nptII
Nicotiana plumbaginifolia ATP synthase gene (atp2-1); β subunit	5' CaMV 35S/atp2-1 signal sequence/cat	tobacco (plants)	+ cat enzyme is targeted into mitochondria
Maize transposable elements Ac and Ds	Ac or Ds within borders of waxy locus	tobacco (tumors and shoots)	+ Ac is capable of self-catalyzed transposition

Abbreviations:
copia LTR - long terminal repeat of copia taansposable element
HSV - Herpes simplex virus
Adh - alcohol dehydrogenase gene
ST-LS 1 - Solanum ruberosum leaf/stem-specific gene
EPSP - 5 -enolpyruvylshikimate-3-phosphate (gene)
lbc3 - member of the leghemoglobin gene family
Ac, Ds - maize transposable elements (activator, dissociation)
rbcS - ribulose-1,5-bisphosphate carboxylase small subunit gene
cab - chlorophyl a/b binding protein gene
nptII - neomycin phosphotransferase II gene
ocs - octopine synthase gene
nos - nopaline synthase gene
cat - chloramphenicol acetyltransferase gene
CaMV 35S, 19S - cauliflower mosaic virus genes encoding 35S and 19S-transcript, respectively
genomic - transferred construct contains the entire gene including 5' and 3' regions
Mode of expression:
+ - correct expression of stably integrated gene
(+) - transient expression, or transcription followed by incorrect processing and/or translation
(−) - gene is not transcribed

The heterologous DNA to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a cotransformation procedure. Both selectable markers and

reporter genes may be flanked with appropriate regulatory sequnces to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Table 2:

genes which confer herbicide resistance or tolerance are also of commercial utility in the resulting transformed plants.

Reporter genes which encode for easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present or expressed by the recipient

TABLE 2

Selectable marker and reporter genes in plant genetic transformation

Gene	Origin	Encoded enzyme	Useful as: Selectable marker	Useful as: Scorable reporter	Resistance against
Neomycin phosphotransferase gene II (nptII)	TnS	neomycin phosphotransferase	++	+	neomycin kanamycin G-418[1]
Neomycin phosphotransferase gene I (nptI)	Nn601	neomycin phosphotransferase	+	+	neomycin kanamycin G-418[2]
Chloramphenicol acetyltransferase gene (cat)	Tn9	chloramphenicol acetyltransferase	(+)	++	chloramphenicol[3]
Bacterial DHPR gene	plasmid R67	dihydrofolate reductase	+	+	methotrexate[4]
Mutated c-DNA of a mouse DHFR gene	mouse	dihydrofolate reductase	++	+	methotrexate[5]
Octopine synthase gene (ocs)	T-DNA	octopine synthase	+	++	toxic opine precursor analogues, i.e. aminoethylcystein[6]
Nopaline synthase gene (nos)	T-DNA	nopaline synthase	-	++	—[7]
Hygromycin phosphotransferase gene (hpt)	*E. coli*	hygromycin phosphotransferase	++	-	hygromycin B[8]
Bicomycin resistance gene	Tn5	?	+	-	bicomycin[9]
Streptomycin phosphotransferase gene	Tn5	streptomycin phosphotransferase	(+)	(+) control plants are not killed by streptomycin	streptomycin[10]
aroA gene	*Salmonella tryphimurium*	EPSP synthase	++	-	glyphosate[11]
bar gene	*Streptomices hygroscopicus*	phosphinothricin acetyltransferase	++	-	phosphinothricin, bialaphos[12]
β-galactosidase gene	*E. coli*	β-galactosidase	-	+	—[13]
Glucuronidase gene (GUS)	*E. coli*	glucuronidase	-	++	—[14]
Bacterial luciterase gene	*Vibrso harvest*	luciferase	-	++	—[15]
Firefly luciferase gene	*Photinus peralis*	luciferase	-	++	—[16]

Only some representative references were chosen in case of the nptII, nos, ocs and cat genes.
Abbreviations
Tn - transpuson
DHFR - dihydrofolate reductase
EPSP synthase - 5-enolpyruvylskikimate-3-phosphate synthase
[1]M. Bevran et al., Nature, 304, 185 (1983); M. DeBlock et al., EMBOJ., 8, 1681 (1984); I. Herrera-Estrella et al., EMBOJ., 2, 987 (1983).
[2]R.T. Fraley et al., PNASUSA, 80, 1803 (1983); H. Pretrzak et al., Nucl, Acids Res., 14, 5857 (1986).
[3]M. DeBlock et al., EMBO J., 3, 1681 (1984); I. Herrera-Estrella et al., Nature, 303, 209 (1983).
[4]N. Brisson et al., Nature, 310, 511 (1984); M. DeBlock et al., ibid., I. Herrera-Estrella et al., EMBO J., 2, 987 (1983).
[5]D.A. Eichholtz et al., Somat. Cell, Mol. Genet., 13, 67 (1987).
[6]G.A. Dahl et al., Theor, Appl. Genet., 66, 233 (1983); H. DeGeve et al., Nature, 300, 752 (1982); A. Hockema et al., Plant Mol. Biol., 5, 85 (1985): M.G. Koztel et al., J. Mol. Appl. Genet., 2, 549 (1981).
[7]J.D.G. Jones et al., EMBO J., 4, 2411 (1985); C.H. Shaw et al., Nuc. Acids Res., 14, 6003 (1986); P. Zambryska et al., EMBO J., 2, 2443 (1983).
[8]A.M. Lloyd et al., Science, 284, 464 (1986); P.I.M. Van den Hazen et al., Plant MOl. Biol., 5, 299 (1985); C. Waldron et al., Plant Mol. Biol., 5, 103 (1985).
[9]J. Hille et al., Plant Mol. Biol., 2, 171 (1986).
[10]J.D.G. Jones et al., Mol. Gen. Genet., 210, 86 (1987).
[11]L. Comai et al., Nature, 317, 741 (1985); J.J. Inllatti et al., Biotechnology, 5, 726 (1987).
[12]M. DeBlock et al., EMBO J., 6, 2513 (1987); C.T. Thompson et al., EMBO J., 6, 2519 (1987).
[13]G. Heimer et al., Biotechnology, 2, 520 (1984).
[14]D.R. Gallic et al., Nuc. Acids Res., 15, 8693 (1987); R.A. Jefferson et al., EMBO J., 6, 1901 (1987).
[15]C. Koncz et al., Mol. Gen. Genet., 204, 383 (1986).
[16]D.W. Ow et al., Science, 234, 856 (1986); D.W. Ow et al., PNAS USA, 84, 4870 (1987); C.D. Riggs et al., Nucl. Acids Res., 15, 8115 (1987).

A preferred selectable marker gene is the hygromycin B phosphotransferase (HPT) coding sequence, which may be derived from *E. coli*. Other selectable markers known in the art include aminoglycoside phosphotransferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin, and G418, as well as those genes which code for resistance or tolerance to glyphosate, methotrexate, imidazolinones, sulfonylureas, bromoxynil, dalapon, and the like. Those selectable marker

organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g. phenotypic change or enzymatic activity. Examples of such genes are provided in Table 2. Preferred genes include the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the beta-glucuronidase gene of the uida locus of *E. coli*, and the luciferase genes from firefly *Photinus pyralis*.

The regulatory sequences useful herein include any constitutive, inducible, tissue or organ specific, or develop-

mental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al, supra. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *Agrobacterium tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase promoter from corn; light inducible promoters such as, ribulose-biphosphate-carboxylase small subunit gene from a variety of species; and the major chlorophyll a/b binding protein gene promoter; 35s and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present on the DNA. Such elements may or may not be necessary for the function of the DNA, although they can provide a better expression or functioning of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the plant. For example, the maize Adh1S first intron may be placed between the promoter and the coding sequence of a particular heterologous DNA. This intron, when included in a DNA construction, is known to generally increase expression in maize cells of a protein. (Callis et al. 1987) However, sufficient expression for a selectable marker to perform satisfactorily can often be obtained without an intron. (Klein et al. 1989) An example of an alternative suitable intron is the shrunken-1 first intron of *Zea mays*. These other elements must be compatible with the remainder of the DNA constructions.

To determine whether a particular combination of DNA and recipient plant cells are suitable for use herein, the DNA may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the DNA has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (GUS) gene (Jefferson et al. 1987). In the case of the microprojectile bombardment transformation process of the present invention, a suitable time for conducting the assay is about 2–3 days after bombardment. The use of transient assays is particularly important when using DNA components which have not previously been demonstrated or confirmed as compatible with the desired recipient cells.

III. DNA Delivery Process

The DNA can be introduced into the regenerable maize callus cultures via a particle bombardment process. A general description of a suitable particle bombardment instrument is provided in Sanford et al. (1987), the disclosure of which is incorporated herein by reference. While protocols for the use of the instrument in the bombardment of maize non-regenerable suspension culture cells are described in Klein et al. (1988a, 1988b, and 1989), no protocols have been published for the bombardment of callus cultures or regenerable maize cells.

In a microprojectile bombardment process, also referred to as a biolistic process, the transport of the DNA into the callus is mediated by very small particles of a biologically inert material. When the inert particles are coated with DNA and accelerated to a suitable velocity, one or more of the particles is able to enter into one or more of the cells where the DNA is released from the particle and expressed within the cell. While some of the cells are fatally damaged by the bombardment process, some of the recipient cells do survive, stably retain the introduced DNA, and express it.

The particles, called microprojectiles, are generally of a high density material such as tungsten or gold. They are coated with the DNA of interest. The microprojectiles are then placed onto the surface of a macroprojectile which serves to transfer the motive force from a suitable energy source to the microprojectiles. After the macroprojectile and the microprojectiles are accelerated to the proper velocity, they contact a blocking device which prevents the macroprojectile from continuing its forward path but allows the DNA-coated microprojectiles to continue on and impact the recipient callus cells. Suitable such instruments may use a variety of motive forces such as gunpowder or shock waves from an electric arc discharge (Swain et al. 1988). An instrument in which gunpowder is the motive force is currently preferred and such is described and further explained in Sanford et al. (1987), the disclosure of which is incorporated herein by reference.

A protocol for the use of the gunpowder instrument is provided in Klein et al. (1988a, b) and involves two major steps. First, tungsten microprojectiles are mixed with the DNA, calcium chloride, and spermidine free base in a specified order in an aqueous solution. The concentrations of the various componenets may be varied as taught. The currently preferred procedure entails exactly the procedure of Klein et al. (1988b) except for doubling the stated optimum DNA concentration. Secondly, in the actual bombardment, the distance of the recipient cells from the end of the barrel as well as the vacuum in the sample chamber. The currently preferred procedure for bombarding the callus entails exactly the procedure of Klein et al. (1988b) with the recipient tissue positioned 5 cm below the stopping plate tray.

The callus cultures useful herein for generation of transgenic plants should generally be about 3 months to 3 years old, preferably about 3 to 18 months old. Callus used for bombardment should generally be about midway between transfer periods and thus past any "lag" phase that might be associated with a transfer to a new media, but also before reaching any "stationary" phase associated with a long time on the same plate.

The specific tissue subjected to the bombardment process is preferably taken about 7–10 days after subculture, though this is not believed critical. The tissue should generally be used in the form of pieces of about 30 to 80, preferably about 40 to 60, mg. The clumps are placed on a petri dish or other surface and arranged in essentially any manner, recognizing that (i) the space in the center of the dish will receive the heaviest concentration of metal-DNA particles and the tissue located there is likely to suffer damage during bombardment and (ii) the number of particles reaching a cell will decrease (probably exponentially) with increasing distance of the cell from the center of the blast so that cells far from the center of the dish are not likely to be bombarded and transformed. A mesh screen, preferably of metal, may be laid on the dish to prevent splashing or ejection of the tissue. The tissue may be bombarded one or more times with the DNA-coated metal particles.

IV. Selection Process

Once the calli have been bombarded with the DNA and the DNA has penetrated some of the cells, it is necessary to identify and select those cells which both contain the heterologous DNA and still retain sufficient regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed

calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively and preferably, when a selectable marker gene has been transmitted along with or as part of the heterologous DNA, those cells of the callus which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells while simultaneously inhibiting the growth of the non-transformed cells. The situation is complicated by the fact that the vitality of individual cells in a population is often highly dependent on the vitality of neighboring cells. Also, the selection conditions must not be so severe that the plant regeneration capacity of the callus cells and the fertility of the resulting plant are precluded. Thus the effects of the selection agent on cell viability and morphology should be evaluated. This may be accomplished by experimentally producing a growth inhibition curve for the given selective agent and tissue being transformed beforehand. This will establish the concentration range which will inhibit growth.

When a selectable marker gene has been used, the callus clumps may be either allowed to recover from the bombardment on non-selective media or, preferably, directly transferred to media containing that agent.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably the concentration of the agent is initially such that about a 5–40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells to preferentially grow and divide while inhibiting untransformed cells, but not to the extent that growth of the transformed cells is prevented. Once the few individual transformed cells have grown sufficiently the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of non-transformed cells habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative maize transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells. The callus may also be cultured on non-selective media at various times during the overall selection procedure.

Once a callus sector is identified as a putative transformant, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e. Southern blot or polymerase chain reaction (PCR) or the like.

V. Regeneration of Plants and Production of Seed

Cell lines which have been shown to be transformed must then be regenerated into plants and the fertility of the resultant plants determined. Transformed lines which test positive by genotypic and/or phenotypic analysis are then placed on a media which promotes tissue differentiation and plant regeneration. Regeneration may be carried out in accordance with standard procedures well known in the art. The procedures commonly entail reducing the level of auxin which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. One example of such a regeneration procedure is described in Green et al. (1981). The plants are grown to maturity in a growth room or greenhouse and appropriate sexual crosses and selfs are made as described by Neuffer (1981).

Regeneration, while important to the present invention, may be performed in any conventional manner. If a selectable marker has been transformed into the cells, the selection agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are transformed. Since regeneration techniques are well known and not critical to the present invention, any technique which accomplishes the regeneration and produces fertile plants may be used.

VI. Analysis of R1 Progeny

The plants regenerated from the transformed callus are referred to as the R0 generation or R0 plants. The seeds produced by various sexual crosses of the R0 generation plants are referred to as R1 progeny or the R1 generation. When R1 seeds are germinated, the resulting plants are also referred to as the R1 generation.

To confirm the successful transmission and inheritance of the heterologous DNA in the sexual crosses described above, the R1 generation should be analyzed to confirm the presence of the transforming DNA. The analysis may be performed in any of the manners such as were disclosed above for analyzing the bombarded callus for evidence of transformation, taking into account the fact that plants and plant parts are being used in place of the callus.

VII. Breeding of Genetically Engineered Commercial Hybrid Seed

Generally, the commercial value of the transformed corn produced herein will be greatest if the heterologous DNA can be incorporated into many different hybrid combinations. A farmer typically grows several varieties of hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based upon his physical location since hybrids adapted to one part of the corn belt are generally not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the heterologous DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable heterologous DNA. This may conveniently be done by breeding programs in which a conversion process (backcrossing) is performed by crossing the initial transgenic fertile plant to normal elite inbred lines and then

crossing the progeny back to the normal parent. The progeny from this cross will segregate such that some of the plants will carry the heterologous DNA whereas some will not. The plants that do carry the DNA are then crossed again to the normal plant resulting in progeny which segregate once more. This crossing is repeated until the original normal parent has been converted to a genetically engineered line containing the heterologous DNA and also possessing all other important attributes originally found in the parent. A separate back-crossing program will be used for every elite line that is to be converted to a genetically engineered elite line. It may be necessary for both parents of a hybrid seed corn to be homozygous for the heterologous DNA. Corn breeding and the techniques and skills required to transfer genes from one line or variety to another are well-known to those skilled in the art. Thus introducing heterologous DNA into lines or varieties which do not generate the appropriate calli can be readily accomplished by these breeding procedures.

VIII. Uses of Transgenic Plants

The transgenic plants produced herein are expected to be useful for a variety of commercial and research purposes.

Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g. agronomic traits such as pest resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g. improved nutritive content in human food or animal feed), or beneficial to the food processor (e.g. improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods. however, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silaage or for ornamental purposes (e.g. Indian corn). often chemical constituents (e.g. oils or starches) of corn and other crops are extracted for food or in dustrial use and transgenic plants may be created which have enhanced or modified levels of such components. The plants may also be used for seed production for a variety of purposes.

Transgenic plants may also find use in the commercial manufacture of proteins or othermolecules encoded by the heterologous DNA contained therein, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules, or for other purposes (e.g. for research).

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded by the heterologous DNA may be transferred, e.g. from corn cells to cells of other species e.g. by protoplast fusion.

The transgenic plants may have many use s in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. The methods of the invention may also be used to create plants having unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties.

The following non-limiting examples are illustrative of the present invention. They are presented to better explain the general procedures which were used to prepare the fertile *Zea mays* plants of this invention which stably express the heterologous DNA and which transmit that DNA to progeny. All parts and percents are by weight unless otherwise specified. It must be recognized that a specific transformation event is a function of the amount of material subjected to the transformation procedure. Thus when individual situations arise in which the procedures described herein do not produce a transformed product, repetition of the procedures will be required.

EXAMPLE I

Fertile transgenic *Zea mays* plants which contain heterologous DNA which is heritable were prepared as follows:

I. Initiation and Maintenance of Maize Cell Cultures which Retain Plant Regeneration Capacity

Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred line A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient agar initiation/maintenance media with the root/shoot axis exposed to the medium. Initiation/maintenance media (hereinafter refered to as F medium) consisted of N6 basal media (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc. San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

The immature embryos were incubated at 26° C. in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh media. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell culture named AB12 used in this example was such a culture and had been initiated about 1 year before bombardment.

II. Plasmids—pCHN1-1, pHYGI1, pBII221, and pLUC-1

The plasmids pCHN1-1, pHYGI1, and pLUC-1 were constructed in the vector pBS+ (Stratagene, Inc., San Diego, Calif.), a 3.2 Kb circular plasmid, using standard recombinant DNA techniques. pCHN1-1 contains the hygromycin B phosphotransferase (HPT) coding sequence from *E. coli* (Gritz et al. 1983) flanked at the 3' end by the nopaline synthase (nos) polyadenylation sequence of Agrobacterium tumefaciens (Chilton and Barnes 1983). Expression is driven by the cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982), located upstream from the hygromycin coding sequence. The plasmid pHYGI1 was constructed by inserting the 553 bp Bcl-BamHI fragment containing the maize Adh1S first intron (Callis et al. 1987) between the CaMV 35S promoter and the hygromycin coding sequence of pCHN1-1. A map of pHYGI1 is provided as FIG. 1.

pBII221 contains the *E. Coli* B-glucuronidase coding sequence flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. The plasmid was constructed by inserting the maize AdhIS first

intron between the 35S promoter and the coding sequence of pBI221 (Jefferson et al. 1987). A map of pBII221 is provided as FIG. 2.

pLUC-1 contains the firefly luciferase coding sequence (DeWet et al. 1987) flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. This plasmid was used solely as a negative control.

Plasmids were introduced into the embryogenic callus culture AB12 by microprojectile bombardment.

III. DNA Delivery Process

The embryogenic maize callus line AB12 was subcultured 7 to 12 d prior to microprojectile bombardment. AB12 was prepared for bombardment as follows. Five clumps of callus, each approximately 50 mg in wet weight were arranged in a cross pattern in the center of a sterile 60×15 mm petri plate (Falcon 1007). Plates were stored in a closed container with moist paper towels throughout the bombardment process. Twenty six plates were prepared.

Plasmids were coated onto M-10 tungsten particles (Biolistics) exactly as described by Klein, et al (1988b) except that, (i) twice the recommended quantity of DNA was used, (ii) the DNA precipitation onto the particles was performed at 0° C., and (iii) the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process.

All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5 M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. When two plasmids were used simultaneously, each was present in an amount of 2.5 ul. One tube contained only plasmid pBII221; two tubes contained both plasmids pHYGI1 and pBII221; two tubes contained both plasmids pCHNl-1 and pBII221; and one tube contained only plasmid pLUC-1.

All tubes were incubated on ice for 10 min., pelletized by centrifugation in an Eppendorf centrifuge at room temperature for 5 seconds, and 25 ul of the supernatant was discarded. The tubes were stored on ice throughout the bombardment process. Each preparation was used for no more than 5 bombardments.

Macroprojectiles and stopping plates were obtained from Biolistics, Inc. (Ithaca, N.Y.). They were sterilized as described by the supplier. The microprojectile bombardment instrument was obtained from Biolistics, Inc.

The sample plate tray was positioned at the position 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate in the slot below the barrel. Plates of callus tissue prepared as described above were centered on the sample plate tray and the petri dish lid removed. A 7×7 cm square rigid wire mesh with 3×3 mm mesh and made of galvanized steel was placed over the open dish in order to retain the tissue during the bombardment. Tungsten/DNA preparations were sonicated as described by Biolistics, Inc. and 2.5 ul was pipetted onto the top of the macroprojectiles. The instrument was operated as described by the manufacturer. The following bombardments were performed:

2×pBII221 prep To determine transient expression frequency

10×pHYGI1/pBII221 As a potential positive treatment for transformation

10×pCHN1-1/pBII221 As a potential positive treatment for transformation

4×pLUC-1 Negative control treatment

The two plates of callus bombarded with pBII221 were transferred plate for plate to F medium (with no hygromycin) and the callus cultured at 26° C. in the dark. After 2 d this callus was then transferred plate for plate into 35×10 mm petri plates (Falcon 1008) containing 2 ml of GUS assay buffer which consists of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (Research Organics), 100 mM sodium phosphate pH 7.0, 5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM EDTA, and 0.06% Triton X-100. These were incubated at 37° C. for 3 d after which the number of blue cells was counted giving 291 and 477 transient GUS expressing cells in the two plates, suggesting that the DNA delivery process had also occurred with the other bombarded plates. These plates were discarded after counting since the GUS assay is destructive.

IV. Selection Process

Hygromycin B (Calbiochem) was incorporated into the medium by addition of the appropriate volume of filter sterilized 100 mg/ml Hygromycin B in water when the media had cooled to 45° C. prior to pouring plates.

Immediately after all samples had been bombarded, callus from all of the plates treated with pHYGI1/pBII221, pCHN1-1/pBII221 and three of the plates treated with pLUC-1 were transferred plate for plate onto F medium containing 15 mg/l hygromycin B, (five pieces of callus per plate). These are referred to as round 1 selection plates. Callus from the fourth plate treated with pLUC-1 was transferred to F medium without hygromycin. This tissue was subcultured every 2–3 weeks onto nonselective medium and is referred to as unselected control callus.

After two weeks of selection, tissue appeared essentially identical on both selective and nonselective media. All callus from eight plates from each of the pHYGI1/pBII221 and pCHN1-1/pBII221 treatments and two plates of the control callus on selective media were transferred from round 1 selection plates to round 2 selection plates that contained 60 mg/l hygromycin. The round 2 selection plates each contained ten 30 mg pieces of callus per plate, resulting in an expansion of the total number of plates.

The remaining tissue on selective media, two plates each of pHYGI1/pBII221 and pCHN1-1/pBII221 treated tissue and one of control callus, were placed in GUS assay buffer at 37° C. to determine whether blue clusters of cells were observable at two weeks post-bombardment. After 6 d in assay buffer this tissue was scored for GUS expression.

TREATMENT	REPLICATE	OBSERVATIONS
pLUC-1		no blue cells
pHYGI1/pBII221	plate 1	11 single cells
		1 four cell cluster
	plate 2	5 single cells
pCHN1-1/pBII221	plate 1	1 single cell
		2 two cell clusters
	plate 2	5 single cells
		1 two cell cluster
		2 clusters of 8–10 cells

After 21 d on the round 2 selection plates, all viable portions of the material were transferred to round 3 selection plates containing 60 mg/l hygromycin. The round 2 selection plates, containing only tissue that was apparently dead, were reserved. Both round 2 and 3 selection plates were observed periodically for viable proliferating sectors.

After 35 d on round 3 selection plates both the round 2 and round 3 sets of selection plates were checked for viable sectors of callus. Two such sectors were observed proliferating from a background of dead tissue on plates treated with pHYGI1/pBII221. The first sector named 3AA was from the round 3 group of plates and the second sector named 6L was

from the round 2 group of plates. Both lines were then transferred to F medium without hygromycin.

After 19 d on F medium without hygromycin the line 3AA grew very little whereas the line 6L grew rapidly. Both were transferred again to F medium for 9 d. The lines 3AA and 6L were then transfered to F medium containing 15 mg/l hygromycin for 14 d. At this point, line 3AA was observed to be of very poor quality and slow growing. The line 6L however grew rapidly on F medium with 15 mg.l hygromycin. In preparation for an inhibition study of the line 6L on hygromycin, the line was then subcultured to F medium without hygromycin.

After 10 d on F medium an inhibition study of the line 6L was initiated. Callus of 6L was transfered onto F medium containing 0, 10, 30, 100, and 250 mg/l hygromycin B. Five plates of callus were prepared f or each concentration and each plate contained ten approximately 50 mg pieces of callus. one plate of unselected control ti ssue was prepared for each concentration of hygromycin.

It was found that the line 6L was capable of sustained growth over 9 subcultures on 0, 10, 30, 100, and 250 mg/l hygromycin. The name of the line 6L was changed at this time from 6L to PH1 (Positive Hlygromycin transformant 1).

Additional sectors were recovered at various time points from the round 2 and 3 selection plates. None of these were able to grow in the presence of hygromycin for multiple rounds, i.e. two or three subcultures.

V. Confirmation of transformed callus

To show that the PHi callus had acquired the hygromycin resistance gene, a Southern blot of PH1 callus was prepared as follows: DNA was isolated from PH1 and unselected control calli by freezing 2 g of callus in liquid nitrogen and grinding it to a fine powder which was transferred to a 30 ml Oak Ridge tube containing 6 ml extraction buffer (7M urea, 250 mM NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1% sarcosine). To this was added 7 ml of phenol:chloroform 1:1, the tubes shaken and incubated at 37° C. 15 min. Samples were centrifuged at 8K for 10 min. at 4° C. The supernatant was pipetted through miracloth (Calbiochem 475855) into a disposable 15 ml tube (American Scientific Products, C3920-15A) containing 1 ml 4.4 M ammonium acetate, pH 5.2. Isopropanol, 6 ml, was added, the tubes shaken, and the samples incubated at −20° C. for 15 min. The DNA was pelleted in a Beckman TJ-6 centrifuge at the maximum speed for 5 min. at 4° C. The supernatant was discarded and the pellet was dissolved in 500 ul TE-10 (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) 15 min. at room temperature. The samples were transferred to a 1.5 ml Eppendorf tube and 100 ul 4.4 M ammonium acetate, pH 5.2 and 700 ul isopropanol were added. This was incubated at −20° C. for 15 min. and the DNA pelleted 5 min. in an Eppendorf microcentrifuge (12,000 rpm). The pellet was washed with 70% ethanol, dried, and resuspended in TE-1 (10 mM Tris-HCl pH 8.0, 1 EM EDTA).

The isolated DNA (10 ug) was digested with BamHI (NEB) and electrophoresed in a 0.8% w/v agarose gel at 15 V for 16 hrs in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The DNA within the gel was then depurinated by soaking the gel twice in 0.25 M HCl for 15 min., denatured and cleaved by soaking the gel twice in 0.5 M NaOH/1.0 M NaCl 15 min., and neutralized by soaking the gel twice in 0.5M Tris pH 7.4/3M NaCl 30 min. DNA was then blotted onto a Nytran membrane (Shleicher & Shuell) by capillary transfer overnight in 6×SSC (20×SSC, 3M NaCl, 0.3M sodium citrate pH 7.0). The membrane was baked at 80° C. for 2 hrs under vacuum. Prehybridization treatment of the membrane was done in 6×SSC, 10×Denhardt's solution, 1% SDS, 50 ug/ml denatured salmon sperm DNA using 0.25 ml prehybridization solution per cm^2 of membrane. Prehybridization was carried out at 42° C. overnight.

A 32P labelled probe was prepared by random primer labelling with an Oligo Labelling Kit (Pharmacia) as per the suppliers instructions with 32P-dCTP (ICN Radiochemicals). The template DNA used was the 1055 bp BamHI fragment of pHYGI1, which is the HPT coding sequence. The fragment was gel purified and cut again with PstI (NEB) before labelling.

The hybridization was performed in 50% formamide, 6×SSC, 1% SDS, 50 ug/ml denatured salmon sperm DNA (Sigma), 0.05% sodium pyrophosphate and all of the isopropanol precipitated heat denatured probe (10^7 CPM/50ug template). The hybridization was carried out at 42° C. overnight.

The membrane was washed twice in 50 ml 6×SSC, 0.1% SDS 5 min. at room temperature with shaking, then twice in 500 ml 6×SSC, 0.1% SDS 15 min. at room temperature, then twice in 500 ml 1×SSC, 1% SDS 30 min. at 42° C., and finally in 500 ml 0.1×SSC 1% SDS 60 min. at 65° C. Membranes were exposed to Kodak X-OMAT AR film in an X-OMATIC cassette with intensifying screens. As shown in FIG. 3, a band was observed for PH1 callus at the expected position of 1.05 Kb, indicating that the HPT coding sequence was present. No band was observed for control callus.

VI. Plant Regeneration and Production of Seed

PH1 callus was transferred directly from all of the concentrations of hygromycin used in the inhibition study to RM5 medium which consists of MS basal salts (Murashige et al. 1962) supplemented with thiamine HCl 0.5 mg/l, 2,4-D 0.75 mg/l, sucrose 50 g/l, asparagine 150 mg/l, and Gelrite 2.5 g/l (Kelco Inc. San Diego).

After 14 d on RM5 medium the majority of PH1 and negative control callus was transferred to R5 medium which is the same as RMT medium, except that 2,4-D is omitted. These were cultured in the dark for 7 d at 26° C. and transferred to a liaht regime of 14 hours light and 10 hours dark for 14 d at 26° C. At this point, plantlets that had formed were transferred to one quart canning jars (Ball) containing 100 ml of R5 medium. Plants were transferred from jars to vermiculite after 14 and 21 d. Plants were grown in vermiculite for 7 or 8 d before transplanting into soil and grown to maturity. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the Ro tissue, a Southern blot was performed as previously described on leaf DNA from three randomly chosen Ro plants of PH1. As shown in FIG. 4, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred lines A188, B73 and Oh43. Seed was harvested 45 days post-pollination and allowed to dry further 1–2 weeks. Seed set varied from 0 to 40 seeds per ear when PH1 was the female parent and from 0 to 32 seeds per ear when PH1 was the male parent.

VII. Analysis of the R1 Progeny

The presence of the hygromycin resistance trait was evaluated by a root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears.

(A) Root Elongation Bioassay

Seed was sterilized in a 1:1 dilution of commercial bleach in water plus alconox 0.1% for 20 min. in 125 ml Erlenmeyer flasks and rinsed 3 times in sterile water and imbibed overnight in sterile water containing 50 mg/ml captan by shaking at 150 rpm.

After imbibition, the solution was decanted from the flasks and the seed transferred to flow boxes (Flow Laboratories) containing 3 sheets of H_2O saturated germination paper. A fourth sheet of water saturated germination paper was placed on top of the seed. Seed was allowed to germinate 4 d.

After the seed had germinated, approximately 1 cm of the primary root tip was excised from each seedling and plated on MS salts, 20 g/l sucrose, 50 mg/l hygromycin, 0.25% Gelrite, and incubated in the dark at 26° C. for 4 d.

Roots were evaluated for the presence or absence of abundant root hairs and root branches. Roots were classified as transgenic (hygromycin resistant) if they had root hairs and root branches, and untransformed (hygromycin sensitive) if they had limited numbers of branches. The results are shown in Table 1.

(B) Etiolated leaf bioassay

After the root tips were excised as described above, the seedlings of one PH1 ear and one control ear were transferred to moist vermiculite and grown in the dark for 5 d. At this point 1 mm sections were cut from the tip of the coleoptile, surface sterilized 10 seconds, and plated on MS basal salts, 20 g/l sucrose, 2.5 g/l Gelrite with either 0 (control) or 100 mg/l hygromycin and incubated in the dark at 26° C. for 18 hr. Each plate contained duplicate sections of each shoot. They were then incubated in a light regimen of 14 hours light 10 hours dark at 26° C. for 48 hr, and rated on a scale of from 0 (all brown) to 6 (all green) for the percent of green color in the leaf tissue. Shoots were classified as untransformed (hygromycin sensitive) if they had a rating of zero and classified as transformed (hygromycin resistant) if they had a rating of 3 or greater. The results are shown in Table 3.

(C) Southern Blots

Seedling form the bioassays were transplanted to sail and are growing to sexual maturity. DNA was isolated from 0.8 g of leaf tissue after about 3 weeks and probed with the HPT coding sequence as described previously. Plants with a 1. 05 Kb band present in the Southern blot were classified as transgenic. As shown in FIG. **5**, two out of seven progeny of PH1 plant 3 were transgeflic as were three out of eight progeny of PHl plant 10. The blot results correlated precisely with data from the bioassays, confirming that the heterologous DNA was transmitted through one complete sexual life cycle. All data are summarized in Table 3.

TABLE 3

ANALYSIS OF PH1 R1 PLANTS

PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.1	+	ND	+	4.1	–	ND	ND
3.2	–	ND	–	4.2	–	ND	ND
3.3	–	ND	–	4.3	–	ND	ND
3.4	–	ND	–	4.4	–	ND	ND
3.5	–	ND	–	4.5	–	ND	ND
3.6	+	ND	+	4.6	–	ND	ND
3.7	–	ND	–	4.7	–	ND	ND
				2.1	–	ND	–
10.1	+	+	+	1.1	–	–	–
10.2	+	+	+	1.2	–	–	ND
10.3	–	–	ND	1.3	–	–	ND
10.4	–	–	–	1.4	–	–	ND
10.5	–	–	–	1.5	–	–	ND
10.6	–	–	–	1.6	–	–	ND
10.7	–	–	–	1.7	–	–	ND
10.8	ND	+	+	1.8	–	–	ND

KEY: + = transgenic; – = nontransgenic; ND = not done

EXAMPLE II

The procedure of Example I was repeated with minor modifications.

I. Plant Lines and Tissue Cultures

The embryogenic maize callus line, AB12, was used as in Example I. The line had been initiated about 18 months before the actual bombardment occurred.

II. Plasmids

The plasmids pBII221 and pHYGI1 described in Example I were used.

III. DNA delivery process

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25, ul 2.5 M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. One tube contained only plasmid pBII221; two tubes contained only plasmid pHYGI1; and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0).

The following bombardments were done:

2 × pBII221 prep	For transient expression
7 × pHYGI1 prep	Potential positive treatment
3 × TE prep	Negative control treatment

After all the bombardments were performed, the callus from the pBII221 treatments was transferred plate for plate to F medium as five 50 mg pieces. After 2 d the callus was placed into GUS assay buffer as per Example I. Numbers of transiently expressing cells were counted and found to be 686 and 845 GUS positive cells, suggesting that the particle delivery process had occurred in the other bombarded plates.

IV. Selection of Transformed Callus

After bombardment the callus from the pHYGI1 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate (different from Example I). The same was done for two of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 13 d the callus on round 1 selection plates was indistinguishable from unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin. An approximate five fold expansion of the numbers of plates occurred.

The callus on round 2 selection plates had increased substantially in weight after 23 d, but at this time appeared close to dead. All of the callus was transfered from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin. This transfer of all material from round 2 to round 3 selection differs from Example I in which only viable sectors were transferred and the round 2 plates reserved.

At 58 d post-bombardment three live sectors were observed proliferating from the surrounding dead tissue. All three lines were from pHYGI1 treatments and were designated 24C, 56A, and 55A.

After 15 d on maintainance medium, growth of the lines was observed. The line 24C grew well whereas lines 55A and 56A grew more slowly. All three lines were transferred to F medium containing 60 mg/l hygromycin. Unselected control callus from maintenance was plated to F medium having 60 mg/l hygromycin.

After 19 d on 60 mg/l hygromycin the growth of line 24C appeared to be entirely uninhibited, with the control showing approximately 80% of the weight gain of 24C. The line 56A was completely dead, and the line 55A was very close. The lines 24C and 55A were transferred again to F 60 mg/l hygromycin as was the control tissue.

After 23 d on 60 mg/l hygromycin the line 24C again appeared entirely uninhibited. The line 55A was completely dead, as was the negative control callus on its second exposure to to F 60 mg/l hygromycin.

At 88 d post-bombardment, a sector was observed proliferating among the surrounding dead tissue on the round 3 selection plates. The callus was from a plate bombarded with pHYGI1 and was designated 13E. The callus was transferred to F medium and cultured for 19 d. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin. After 14 d of culture, the callus line 13E appeared uninhibited on both levels of hygromycin. The control callus appeared to have about 80% of the weight gain of 13E. The callus lines were transferred to fresh media at the same respective levels of hygromycin.

V. Confirmation of Transformed Callus

A Southern blot was prepared from DNA from the line 24C. As shown in FIG. 6, a band was observed for the line 24C at the expected size of 1.05 Kb showing that the line 24C contained the HPT coding sequence. No band was observed for DNA from control tissue. The name of the callus line 24C was changed to PH2.

VI. Plant Regeneration and Production of Seed

The line 24C along with unselected control callus were placed onto RM5 medium to regenerate plants as in Example I. After 16 d the callus was transferred to R5 medium as in Example I.

EXAMPLE III

The procedure of Example II was repeated exactly except that different plasmids were used.

The plasmids pBII221 and pHYGI1 described in Example I were used as well as pMS533 which is a plasmid that contains the insecticidal Bacillus thuringiensis endotoxin (BT) gene fused in frame with the neomycin phosphotransferase (NPTII) gene. 5' of the fusion gene are located segments of DNA from the CaMV 35S and nopaline synthase promoters. 3' from the fusion gene are segments of DNA derived from the tomato protease inhibitor I gene and the poly A region of the nopaline synthase gene.

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. Two tubes contained plasmids pHYGI1 and pMS533 and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0).

The following bombardments were done:

9×pHYGI1/pMS533 Potential positive treatment

2 ×TE prep Control treatment

After bombardment the callus from the pHYGI1/pMS533 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate. The same was done for one of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 12 d the callus on round 1 selection plates appeared to show about 90% of the weight gain of the unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin as ten 30 mg pieces per plate.

After 22 d of selection on round 2 selection plates, the callus appeared completely uninhibited. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin.

At 74 d post-bombardment a single viable sector was observed proliferating from the surrounding necrotic tissue. The callus line was from pHYGI1/pMS533 treated material and was designated 86R. The callus line 86R was transferred to F medium.

After 24 d the callus line 86R had grown substantially. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin.

After 19 d of culture, the callus line 86R appeared to grow rapidly and was uninhibited on both levels of hygromycin. The control callus appeared to have only about 50% of the weight gain of 86R. The callus lines were transferred to fresh media at the same respective levels of hygromycin to further test the resistance of the callus line 86R to hygromycin.

COMPARATIVE EXAMPLE A

The basic procedures of Examples I–III have been attempted except varying the selection regime or the form of the callus. These other attempts, which are detailed in Table 4 below, were not successful. Since they were not repeated several times, it is not known whether they can be made to

work. In all of the procedures, no viable sectors were observed. In the Table, "Sieved" indicates that the callus was passed through an 860 micron sieve before bombardment; the selective agent was hygromycin for each case except when pMXTI1 was the plasmid and methotrexate the selection agent.

TABLE 4

Summary of Comparative Example A

Recip. Tissue	Plasmids	Recov. Period	Round 1 Level	Round 1 Period	Round 2 Level	Round 2 Period
Clumps	pCHN1-1 pBII221	13	60	21	60	81
Clumps	pCHN1-1 pBII221	14	100	22	—	—
Clumps	pHYGI1 pBII221	8	60	19	30	132
Clumps	pCHN1-1 pBII221	0	30	22	60	109
Clumps	pMTXI1 pBII221	8	3	103	—	—
Sieved	PCHN1-1 pBII221	13	—	—	—	—

What is claimed is:

1. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, wherein said DNA comprises at least a screenable marker gene; (ii) selecting a population of transformed cells expressing the selectable marker gene; and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is expressed so as to impart glyphosate resistance to said transgenic plant and is transmitted through a normal sexual cycle of said transgenic plant to progeny plants.

2. The process of claim 1 wherein the fertile transgenic *Zea mays* plant is regenerated from transformed embryogenic tissue.

3. The process of claim 1 wherein the cells are derived from immature embryos.

4. The process of claim 1 wherein said DNA encodes an EPSP synthase.

5. The process of claim 1 further comprising obtaining transgenic glyphosate resistant progeny plants of subsequent generations from said fertile transgenic plant.

6. The process of claim 5 further comprising obtaining seed from one of said progeny plants.

7. A process for producing seed comprising:

(a) providing a fertile transgenic *Zea mays* produced by the process of claim 1, plant comprising heterologous heritable DNA that is expressed so as to impart glyphosate resistance to said transgenic *Zea mays* plant;

(b) cultivating said transgenic *Zea mays* plant;

(c) obtaining seed from said cultivated *Zea mays* plant.

8. The process of claim 7 wherein the DNA encodes an EPSP synthase.

* * * * *

EXHIBIT E

US005554798A

United States Patent [19]

Lundquist et al.

[11] Patent Number: 5,554,798

[45] Date of Patent: Sep. 10, 1996

[54] FERTILE GLYPHOSATE-RESISTANT TRANSGENIC CORN PLANTS

[75] Inventors: **Ronald C. Lundquist**, Minnetonka; **David A. Walters**, Bloomington, both of Minn.

[73] Assignee: **DeKalb Genetics Corporation**, St. Paul, Minn.

[21] Appl. No.: **441,073**

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Related U.S. Application Data

[63] Continuation of Ser. No. 508,045, Apr. 11, 1990, Pat. No. 5,484,956, which is a continuation-in-part of Ser. No. 467,983, Jan. 22, 1990, abandoned.

[51] **Int. Cl.**[6] **A01H 4/00**; C12N 15/05

[52] **U.S. Cl.** **800/205**; 800/DIG. 56; 435/172.3; 435/172.1; 435/240.5; 435/240.45; 536/23.71

[58] **Field of Search** 800/205, 250, 800/DIG. 56; 435/172.3, 172.1

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,370,160	1/1983	Ziemelis	71/117
4,399,216	8/1983	Axel et al.	435/6
4,535,060	8/1985	Comai	435/172.3
4,559,301	12/1985	Turner	435/76
4,559,302	12/1985	Ingolia	435/172.3
4,581,847	4/1986	Hibberd et al.	47/58
4,634,665	1/1987	Axel et al.	435/68
4,642,411	2/1987	Hibberd et al.	800/1
4,665,030	5/1987	Close	435/240
4,666,844	5/1987	Cheng	435/240
4,683,202	7/1987	Mullis	435/91
4,727,028	2/1988	Santerre et al.	435/240.2
4,743,548	5/1988	Crossway et al.	435/172.3
4,761,373	8/1988	Anderson et al.	435/172.3
4,806,483	2/1989	Wang	435/240.49
4,940,835	7/1990	Shah et al.	800/205
4,971,908	11/1990	Kishore et al.	435/172.1
5,001,060	3/1991	Peacock et al.	435/172.3
5,004,863	4/1991	Umbeck	800/205
5,015,580	5/1991	Christou et al.	435/172.3
5,034,322	7/1991	Rogers et al.	435/172.3
5,049,500	9/1991	Arnizen et al.	435/172.3
5,094,945	3/1992	Comai	435/172.3
5,110,732	5/1992	Benfey et al.	435/172.3
5,134,074	7/1992	Gordon et al.	435/240.4
5,177,010	1/1993	Goldman et al.	435/172.3
5,187,073	2/1993	Goldman et al.	435/172.3
5,188,642	2/1993	Shah et al.	47/58
5,188,958	2/1993	Moloney et al.	435/240.4
5,250,515	10/1993	Fuchs et al.	514/12
5,254,799	10/1993	DeGrave et al.	800/205
5,258,300	11/1993	Glassman et al.	435/240.4
5,268,463	12/1993	Jefferson	536/23.7
5,290,924	3/1994	Last et al.	536/24.1
5,302,523	4/1994	Coffee et al.	435/172.1
5,350,689	9/1994	Shillito et al.	435/240.47
5,352,605	11/1994	Fraley et al.	435/240.4
5,371,003	12/1993	Murray et al.	435/172.3
5,405,765	4/1995	Vasil et al.	435/172.3

FOREIGN PATENT DOCUMENTS

80893/87	12/1988	Australia	C12N 15/00
0126537A2	4/1983	European Pat. Off.	A61K 9/52
0131623B1	1/1984	European Pat. Off.	C12N 15/11
0141373A3	5/1985	European Pat. Off.	A01G 7/00
0154204A2	9/1985	European Pat. Off.	C12N 15/00
0160390A2	11/1985	European Pat. Off.	A01H 15/10
0193259A1	9/1986	European Pat. Off.	C12N 15/00
0204549A2	10/1986	European Pat. Off.	C12N 15/00
0202668A2	11/1986	European Pat. Off.	C12N 5/02
0242236A1	10/1987	European Pat. Off.	C12N 15/00
0242246A1	11/1987	European Pat. Off.	C12N 15/00
0299552A1	1/1988	European Pat. Off.	C12N 15/00
0257472A2	3/1988	European Pat. Off.	C12N 15/00
0262971A2	5/1988	European Pat. Off.	A01H 1/02
0271408	6/1988	European Pat. Off.	C12N 15/00
0270356A2	6/1988	European Pat. Off.	C12N 15/00
0275069A2	7/1988	European Pat. Off.	C12N 15/00
0280400A2	8/1988	European Pat. Off.	A01C 1/06
0282164A2	9/1988	European Pat. Off.	C12N 5/00
0292435A1	11/1988	European Pat. Off.	C12N 15/00
0289479A2	11/1988	European Pat. Off.	C12N 15/00
0290395A2	11/1988	European Pat. Off.	C12N 15/00
0301749A2	2/1989	European Pat. Off.	C12N 15/00
0353908A2	7/1989	European Pat. Off.	C12N 15/29
0334539A2	9/1989	European Pat. Off.	C12N 15/00
0331855A2	9/1989	European Pat. Off.	C12M 3/00
0348348A2	12/1989	European Pat. Off.	A01N 65/00
0385962A1	2/1990	European Pat. Off.	C12N 15/82
0360750A2	3/1990	European Pat. Off.	C12N 15/29
0359617A2	3/1990	European Pat. Off.	C12N 15/53
0408403A1	5/1990	European Pat. Off.	C12N 15/32
0442174A1	4/1991	European Pat. Off.	C12N 15/82
0424047A1	4/1991	European Pat. Off.	C12N 15/87
0459643A2	5/1991	European Pat. Off.	C12N 15/82
0442175A1	8/1991	European Pat. Off.	A01H 1/02
0452269A2	11/1991	European Pat. Off.	C12N 15/82
0469273A1	2/1992	European Pat. Off.	C12N 15/82
0485970A3	5/1992	European Pat. Off.	C12N 15/82

(List continued on next page.)

OTHER PUBLICATIONS

"Dekalb Researchers Produce Fertile Corn Plants with Foreign Genes," *ARI Newsletter* (Oct./Nov. 1990).

"Genetic Engineering Advance Announced for Corn Plants," Investor's Daily, (Apr. 19, 1990).

"Genetically Engineered Corn: Breakthrough Brings Market Closer," *Genetic Technology News*, 8–11 (Oct. 1990).

"Keystone Crops," *Agricultural Genetics Report*, (Mar./Apr. 1990).

(List continued on next page.)

Primary Examiner—Gary Benzion

Attorney, Agent, or Firm—Schwegman, Lundberg, Woessner & Kluth, P.A.

[57] **ABSTRACT**

Fertile transgenic *Zea mays* (corn) plants which stably express heterologous DNA which is heritable are provided along with a process for producing said plants. The preferred process comprises the microprojectile bombardment of friable embryogenic callus from the plant to be transformed. The process may be applicable to other graminaceous cereal plants which have not proven stably transformable by other techniques.

6 Claims, 6 Drawing Sheets

FOREIGN PATENT DOCUMENTS

0589110A1	3/1994	European Pat. Off.	A01N 63/02
2661421	11/1991	France	C12N 15/09
0018970	9/1882	Germany .	
3738874A1	11/1988	Germany	A01H 1/06
4013099A1	10/1991	Germany	C12N 15/82
61-134343	5/1984	Japan .	
8801444	1/1990	Netherlands	C12N 15/87
2159173	11/1985	United Kingdom	C12N 15/00
WO85/01856	5/1985	WIPO	A01B 76/00
WO85/02973	7/1985	WIPO	A01J 7/00
WO85/02972	7/1985	WIPO	A01C 1/06
WO87/04181	7/1987	WIPO	C12N 1/00
WO87/05629	9/1987	WIPO	C12N 15/00
WO89/04371	5/1989	WIPO	C12N 21/00
WO89/12102	12/1989	WIPO	C12N 15/00
WO89/11789	12/1989	WIPO	A01H 1/00
WO90/01869	3/1990	WIPO	A01H 1/00
WO90/02801	3/1990	WIPO	C12N 15/32
WO90/10691	8/1990	WIPO	C12N 5/00
WO90/10725	9/1990	WIPO	C23C 16/00
WO91/02071	2/1991	WIPO	C12N 15/82
WO91/04323	4/1991	WIPO	C12N 9/10
WO91/00183	5/1991	WIPO .	
WO91/10725	7/1991	WIPO	C12N 5/00
WO91/16432	10/1991	WIPO	C12N 15/31
WO92/06205	4/1992	WIPO	C12N 15/82
WO92/09696	6/1992	WIPO	C12N 15/82
WO92/12250	7/1992	WIPO	C12N 15/82
WO92/19731	11/1992	WIPO	C12N 15/00
WO93/07278	4/1993	WIPO	C12N 15/82
WO93/08682	5/1993	WIPO	A01H 1/00
WO93/14210	7/1993	WIPO	C12N 15/82
WO93/19190	9/1993	WIPO	C12N 15/82
WO93/21335	10/1993	WIPO	C12N 15/87

OTHER PUBLICATIONS

Chan, M.–T., et al., "Agrobacterium–Mediated Production of Transgenic Rice Plants Expressing a Chimeric α–Amylase Promoter/β–Glucuronidase Gene," Plant Mol. Biol., 22, 491–506 (1993).

Hiei, Y., et al., "Efficient Transformation of Rice (*Oryza sativa* L.) Mediated by Agrobacterium and Sequence Analysis of the Boundaries of the T–DNA," The Plant J., 6, 271–282 (1994).

Abstract, 35th Annual Maize Genetics Conference, *In Vitro Cellular and Devel. Biol.*, 28:(3) (1992).

"Bullets Transform Plant Cells," *Agricell Report*, 9, 5, (Jul. 1987).

"BioTechnica Applies for Field Test of Genetically Engineered Corn," *Genetic Technology News*, 10(3), (Mar. 1990).

Catalog, *Handbook of Fine Chemicals*, Aldrich Chem. Co., p. 508 (1988).

"Chipping Away at Old Weed Enemies," Farm Science Outlook, *Prairie Farmer* 162, 34 (Feb. 20 1990).

"Corn Transformers Multiply," *Bio/Technol.*, 8, 490 (Jun. 1990).

"Cornell U. Gene Gun Hits Biotech Bullseye," *Agriculture Technology*, p. 13.

"Dalapon," Merck Index, 11th edition, S. Budavae, (ed.), Merck and Co., pp. 405–406 (1989).

Dialog Search of Japanese Patent No. 61–134343 (1986).

EPO Notice Regarding Publication of Bibliographic Data for EPO 0485506 (1992).

"Genes Guns Succeed in Altering Corn," *Biotechnology News*, p. 2 (Apr. 1990).

"Herbicide–Resistant Corn" *CT Academy of Science and Engineering, Case Reports*, 5(4), 6 (1990).

International Search Report, PCT/US 90/04462, mailed Jan. 15, 1991.

International Search Report, PCT/US 90/09699, mailed Aug. 16, 1995.

International Society for Plant Molecular Biology, Program and Abstract, Molecular Biology of Plant Growth and Development, Tuson, Arizona, Oct. 6–11 (1991).

Patent Family Record for Australian Patent 87 80 893.

"Plant Science Research, Inc. Achieves Successful Transformation of Corn," *Genetic Engineering News*, 10(3), 3 (March 1990).

"Shotgunning DNA into Cells," *Genetic Engineering News*, (Jul./Aug. 1987).

"Sticky Ends," *Genetic Engineering News*, 10(5), 1 (May 1990).

"Teams from USDA/Monsanto and DeKalb Genetically Engineer Corn," *Genetic Technology News*, 10(5) (May 1990).

"Two Teams Succeed in Putting Foreign Genes in Corn Plants," *Genetic Engineering Letter*, 10(8), 3 (Apr. 24, 1990).

"USDA Approves More Field Tests," *Genetic Technology News*, 11(7), 12 (Jul. 1991).

"USDA Approves Field Test for BioTechnica's Genetically Engineered Corn," *Genetic Technology News*, 10(7), 6 (Jul. 1990).

Adang, M. J., et al., "Characterized Full–Length and Truncated Plasmid Clones of the Crystal Protein of *Bacillus thuringiensis* subsp. kurstaki HD–73 and Their Toxicity to *Manduca sexta*," *Gene*, 36, 289–300 (1985).

Ahokas, H. "Transfection of Germinating Barley Seed Electrophoretically with Exogenous DNA," *Theor. Appl. Genet.*, 77, 469–472 (1989).

Ahokes, H. "Electrophoretic transfection of cereal grains with exogenous nucleic acid," Soc. Biochem. Biophys. Microbio. Fen., Biotieteen Paivat (Bioscience Days), Abstracts, Technical University of Helsinki, Espoo, p. 2 (1989).

Akella, V., et al., "Expression in Cowpea Seedings of Chimeric Transgene after Electroporation into Seed–Derived Embryos," *Plant Cell Rep.*, 12, 110–117 (1993).

Altenbach, S. B., et al., "Enhancement of the Methionine Content of Seed Proteins by the Expression of a Chimeric Gene Encoding a Methionine–Rich Protein in Transgenic Plants," *Plant. Mol. Biol.*, 13, 513–522 (1989).

Altenbach, S. B., et al., "Cloning and Sequence Analysis of a cDNA Encoding a Brazil Nut Protein Exceptionally Rich in Methionine," *Plant Mol. Biol.*, 8, 239–250 (1987).

Ampe, C., et al., "The Amino–Acid Sequence of the 2S Sulphur–Rich from Seed of Brazil Nut (*Bertholletia excelsa* H.B.K.)," *Eur. J. Biochem.*, 159, 597–604 (1986).

Armstrong, C. L., et al., "Establishment and Maintenance of Friable, Embryogenic Maize Callus and the Involvement of L–Proline," *Planta*, 164, 207–214 (1985).

Armstrong, C. L., et al., "Genetic and cytogenetic variation in plants regenerated from organogenic and friable, embryonic tissue cultures of maize," *Biological Abstracts*, vol. 85, Abstract No. 117662 (1988).

Aves, K., et al., "Transformation of an Elite Maize Inbred Through Microprojectile Bombardment of Regenerable Embryonic Callus", *In Vitro Cell. Develop. Biol.*, 28A, p. 124A, Abstract No. P–1134 (1992).

Bao-Jian, L., et al., "Introduction of Foreign Genes into the Seed Embryo Cells of Rice by Electroinjection and the Regeneration of Transgenic Rice Plants," *Science in China*, 34, 925–931 (1991).

Barker, R. F., et al., "Nucleotide Sequence of the T–DNA Region from the *Agrobacterium tumefaciens* Octopone Ti Plasmid pTil5955," *Plant Mol. Biol.*, 2, 335–350 (1983).

Beerman, F., et al., "Tyrosinase as a Marker for Transgenic Mice," *Nuc. Acids. Res.*, 19, 958 (1991).

Belanger, F. C., et al., "Molecular Basis for Allelic Polymorphism of the Maize Globulin–1 Gene" *Genetics*, 129, 863–872 (1991).

Benner, M. S., et al., "Genetic Analysis of Methionine–Rich Storage Protein Accumulation in Maize," *Theor. Appl. Genet.*, 78, 761–767 (1989).

Bevan, M., et al., "A Chimaeric Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation," *Nature*, 304, 184–187 (1983).

Bevan, M., et al., "Structure and Transcription of the Nopaline Synthase Gene Region of T–DNA," *Nuc. Acids Res.*, 11, 369–385 (1983).

Binns, A. N., "Agrobacterium–mediated gene delivery and the biology of host range limitations," *Physiologia Plantarium*, 79, 135–139 (1990).

Bishop, J. E., "Two Teams Plant Genes into Corn," *The Wall Street Journal*, B1 (Apr. 1990).

Booy, G., et al., "Attempted Pollen—Mediated Transformation of Maize," *J. Plant Physiol.*, 135, 319–324 (1989).

Boulton, M. I., et al., "Specificity of *Agrobacterium*—mediated delivery of maize streak virus DNA to members of the Gramineae," *Plant Molecular Biology*, 12, 31–40 (1989).

Brill, W. J., "Agricultural Microbiology," *Scientific American*, 245(3), 199–215 (Sep. 1981).

Brunke, K. J., et al., "Insect Control with Genetically Engineered Crops," *Trends in Biotechnol.*, 9, 197–200 (1991).

Buchanan–Wollaston, V., et al., "Detoxification of the Herbicide Dalapon by Transformed Plants," *J. of Cell. Biochem.*, 13D, p. 330, Abstract No. M503 (1989).

Callis, J., et al., "Introns Increase Gene Expression in Cultures Maize Cells," *Genes and Development*, 1, 1183–1200 (1987).

Cao, J., et al., "Transformation of Rice and Maize Using the Biolistic Process," In: *Plant Gene Transfer*, Alan R. Liss, Inc., pp. 21–33 (1990).

Carpita, N. C., "The Biochemistry of "Growing" Cell Walls," In: *Physiology of Cell Expansion During Plant Growth*, Cosgrove, D. J., et al., (eds.) Am. Soc. Plant Physiol., pp. 28–100 (1987).

Chandler, V. L., et al., "Two Regulatory Genes of the Maize Anthocyanin Pathway are Homologous: Isolation of B Utilizing R Genomic Sequences," *The Plant Cell*, 1, 1175–1183 (1989).

Chasan, R., "Transforming Maize Transformation," *The Plant Cell*, 4, 1463–1464 (1992).

Chourey, P. S., et al., "Callus Formation from Protoplasts of a Maize Cell Culture," *Theor. Appl. Genet.*, 59, 341–344 (1981.

Christou, P., et al., "Opine Synthesis in Wild—Type Plant Tissue," *Plant Physiol.*, 82, 218–221 (1986).

Christou, P., et al., "Soybean Genetic Engineering—Commercial Production of Transgenic Plants," *Trends Biotechnol.*, 8, 145–151 (1990).

Christou, P., et al., "Cotransformation Frequencies of Foreign Genes in Soybean Cell Cultures," *Theor. Appl. Genet.*, 79, 337–341 (1990).

Christou, P., et al., "Genetic Transformation of Crop Plants Using Microprojectile Bombardment," *The Plant Journal*, 2, 275–281 (1992).

Christou, P., et al., "Stable Transformation of Soybean Callus by DNA—Coated Gold Particles," *Plant Physiol.*, 87, 671–674 (1988).

Chu, C.—C., et al., Establishment of an Efficient Medium for Anther Culture of Rice Through Comparative Experiments on the Nitrogen Sources," *Sci. Sin. (Peking)*, 13, 659–668 (1975).

Clark, B., "Biotech Advance in Corn: Gunslinging Researchers Fire Marker Genes in to Corn," *AG Consultant*, 46(7), 12(Jul. 1990).

Cocking, F., et al., "Gene Transfer in Cereals," *Science*, 236, 1259–1262 (1987).

Coe et al., "The Genetics of Corn" In: *Corn and Corn Improvement*, 2nd edition, Sprague, G. F., (ed.), American Soc. Agronomy, Inc, Madison, WI, p. 138 (1977).

Comai, L., et al., "Expression in Plants of a Mutant aroA Gene from *Salmonella typhimurium* Confers Tolerance to Glyphosate," *Nature*, 317, 741–744 (Oct., 1985).

Creissen, G., et al., "Agrobacterium— and Microprojectile—Mediated Viral DNA Delivery into Barley Microspore Derived–Cultures," *Plant Cell Rep.*, 8, 680–683 (Apr. 1990).

Crossway, A., et al., "Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts," *Mol. Gen. Genet.*, 202, 179–185 (1986).

D'Halluin, K., et al., "Transgenic Maize Plants by Tissue Electroporation," *The Plant Cell*, 4, 1495–1505 (1992).

Darvill, A., et al., "The Primary Cell Walls of Flowering Plants," In: *The Biochemistry of Plants*, vol. 1, pp. 91–162 (1980).

Dauce–LeReverand, B., et al., "Improvement of *Escherichia coli* Strains Overproducing Lysine Using Recombinant DNA Techniques," *Eur. J. Appl. Microbiol. Biotechnol.*, 15, 227–231 (1982).

De Block, M., et al., "Engineering herbicide resistance on plants by expression of a detoxifying enzyme," *EMBO J.*, 6, 2513–2518 (1987).

De Greef, W., et al., "Evaluation of herbicide resistance in transgenic crops under field conditions," *Bio/Technol.*, 7, 61–64 (1989).

Dekeyser, R. A., et al., "Evaluation of Selectable Markers for Rice Transformation," *Plant Physiol.*, 90, 217–223 (1989).

Dekeyser, R. A., et al., "Transient Gene Expression in Intact and Organized Rice Tissues," *The Plant Cell*, 2, 591–602, (1990).

DeWald et al., "Plant regeneration from inbred maize suspensions," VIIth International Congress on Plant Tissue and Cell Culture, p. 12, Abstract No. A1–36 (Jun. 24–29, 1990).

DeWet, J. M. J., et al.; "Exogenous gene transfer in maize (*Zea mays*) using DNA–treated pollen;" In: *The experimental manipulation of ovule tissues*. Chapman, G. P., et al., (eds.), Longman, New York, pp. 197–209 (1985).

DeWet, J. R. et al., "Cloning of Firefly Luciferase cDNA and the Expression of Active Luciferase in *Escherichia coli*," *Proc. Nat. Acad. Sci. USA*, 82, 7870–7873 (1985).

Donn, G., et al., "Stable Transformation of Maize with a Chimaeric, Modified Phosphinothricin-Acetyltransferase Gene from *Streptomyces viridochromogenes*," Abstracts, VIIth International Congress Plant Tissue Cell Culture, p. 53, Abstract No. A2-38 (Jun. 24-29, 1990).

Dupuis, I., et al., "Gene Transfer to Maize Male Reproductive Structure by Particle Bombardment of Tassel Primordia," *Plant Cell Rep.*, 12, 607 (1993).

Ellis, J. G., et al., "Does the OCS-Element Occur as a Functional Component of the Promotors of Plant Genes?" *EMBO J.*, 6, 3203-3208 (1987).

Evans, D. A., et al., "Somaclonal Variation—Genetic Basic and Breeding Applications," *Trends Genet.*, 5, 46-50 (1989).

Fennel, A., et al., "Electroporation and PEG Delivery of DNA into Maize Microspores," *Plant Cell Reports*, 11, 567-570 (1992).

Fitzpatrick, T., "Pleiotrophic Gene Found in Barley Plant," *Genetic Engineering News*, 13, 1 (1993).

Fransz, P., et al., "Cytodifferentiation during callus initiation and somatic embryogenesis in *Zea mays* L.," Ph.D. thesis, U. of Wageningen Press, The Netherlands (1988).

Freeling, J. C., et al., "Developmental Potentials of Maize Tissue Cultures," *Maydica*, XXI, 97-112 (Jul. 1977).

Freiberg, "More Researchers Discover Corn Transformation Technology," *AG Biotechnology News*, p. 26 (1990).

Fromm, M. E., et al., "Inheritance and Expression of Chimeric Genes in the Progeny of Transgenic Maize Plants," *Bio/Technol.*, 8, 833-839 (1990).

Fromm, M. E., et al., "Stable Transformation of Maize after Gene Transfer by Electroporation," *Nature*, 319, 791-793 (1986).

Fromm, M., et al., "Expression of Genes Transfected into Monocot and Dicot Plant Cells by Electroporation," *Proc. Nat. Acad. Sci. USA*, 82, 5824-5828 (1985).

Fry, S. C., "Introduction to the Growing Cell Wall," In: *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*, Longman Scientific and Technical, New York, pp. 1-5, 102-109 (1988).

Geiser, M., et al., "The Hypervariable Region on the Genes Coding for Entomopathogenic Crystal Proteins of *Bacillus thuringiensis:* Nucleotide Sequence of the *kurhdl* gene of subsp. *kurstaki* HD1," *Gene*, 48, 109-118 (1986).

Goff, S. A., et al., "Plant Regeneration of Anthocyanin Biosynthetic Genes Following Transfer of B Regulatory Genes into Maize Tissues," *EMBO J.*, 9, 2517-2522 (1990).

Gordon-Kamm, W. J., et al., "Stable Transformation of Embryonic Maize Cultures by Microprojectile Bombardment," *J. Cellular Biochem.*, 13D, p. 259, Abstract No. M122 (1989).

Gordon-Kamm, W. J., et al., "Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants," *The Plant Cell*, 2, 603-618 (1990).

Gould, J., et al., "Transformation of the Graminae by *Agrobacterium tumefaciens*," Int. Soc. Plant Mol. Biol. 3rd Int. Congress, Abstract No. 1277 (1991).

Gould, O., et al., "Shoot Tip Culture as a Potential Transformation System," Abstracts, Beltwide cotton production research conferences, New Orleans, LA, p. 91 (1988).

Gould, J., et al., "Transformation of *Zea mays* L. Using *Agrobacterium tumefaciens* and the Shoot Apex," *Plant Physiol.* 95, 426-434 (1991).

Graves, A., et al., "The Transformation of *Zea mays* seedings with *Agrobacterium tumefacians*," *Plant Mol. Biol.*, 7, 43-50 (1986).

Green, C., et al., "Plant Regeneration from Tissue Cultures of Maize," *Crop. Sci.*, 15, 417-421 (1975).

Green, C., et al., "Plant Regeneration in Tissue Cultures of Maize," In: *Maize for Biological Research*, Sheridan, W. F., (ed.) Plant Mol. Biol. Assoc., pp. 367-372 (1982).

Green, C., et al., "Somatic Cell Genetic Systems, in Corn," In: *Advances in Gene Technology: Molecular Genetics of Plant and Animals*, Academic Press, Inc., pp. 147-157 (1983).

Grimsley, N., et al., "DNA Transfer from Agrobacterium to *Zea mays* or *Brassica* by Agroinfection is Dependent on Bacterial Virulence Functions," *Mol. Gen. Genet.*, 217, 309-316 (1989).

Gritz, L., et al., "Plasmid—Encoded Hygromycin B Resistance: The Sequence of Hygromycin B Phosphotransferase Gene and Its Expression in *Escherichia coli* and *Saccharomyces cerevisiae*, " *Gene*, 25, 179-188 (1983).

Guerineau, F., et al., "Sulfonamide Resistance Gene for Plant Transformation," *Plant Molecular Biology*, 15, 127-136 (1990).

Guilley, H., et al., "Transcription of Cauliflower Mosaic Virus DNA: Detection of Promoter Sequences, and Characterization of Transcripts," *Cell*, 30, 763-773 (Oct. 1982).

Gunset, G., "Genetic Advance May Transform Corn," *Chicago Tribune* (Apr. 19, 1990).

Gunset, G., "Corn Farmers See Economic, Environmental Gold in Designer Genes," *Chicago, Tribune* (Jan. 21, 1991).

Hallauer, A. R., et al., "Corn Breeding," In: *Corn and Corn Improvement*, 3rd edition, Sprague, G. F., et al., (eds.), Agronomy Soc. Amer., pp. 463-564 (1988).

Haughn, G. W., "Transformation with a Mutant Arabidopsis Acetolactate Synthase Gene Renders Tobacco Resistant to Sulfonylurea Herbicides," *Mol. Gen. Genet.*, 211, 266-271 (1988).

Hautpman, R. M., et al., "Evaluation of Selectable Markers for Obtaining Stable Transformants on the Gramineae," *Plant Physiol.*, 86, 602-606 (1988).

Hoffman, L. M., et al., "A Modified Storage Protein is Synthesized, Processed, and Degraded in the Seeds of Transgenic Plants," *Plant Mol. Biol.*, 11, 717-729 (1988).

Hoffman, L. M., et al., "Synthesis and Protein Body Deposition of Maize 15kD Zein in Transgenic Tobacco Seeds," *EMBO J.*, 6, 3213-3221 (1987).

Hofte, H., et al., "Insecticidal Crystal Proteins of *Bacillus thuringiensis," Microbiol. Rev.*, 53, 242-255 (1989).

Hong, B., et al., "Developmental and Organ—Specific Expression of an ABA—and Stress-Induced Protein in Barley," *Plant Mol. Biol.*, 18, 663-674 (1992).

Hooykaas, P. J. J., "Transformation of plant cell via Agrobacterium," *Plant Mol. Biol.*, 13, 327-336 (1989).

Horn, M., et al., "Transgenic Plants of Orchard Grsss (*Dactylis glomerata L.*) from Protoplasts," *Chem. Abstracts*, 110, p. 208, Abstract no. 89869a (1989).

Horn, M., et al., "Transgenic Plants of Orchard grass (*Dactylis glomerata L.*) from Protoplasts," *Plant Cell Reports*, 7, 469 (1988).

Howe, A., et al., "Development of Glyphosphate as a Selectable Marker for the production of Fertile Transgenic Corn Plants," *In Vitro Cell Develop. Biol.*, 28A, p. 124A, Abstract No. P-1136 (Jul.-Aug. 1992).

Huang, Y., et al., "Factors Influencing Stable Transformation of Maize Protoplasts by Electroporation," *Plant Cell, Tissue and Organ Culture*, 18, 281 (1989).

Imbrie-Milligan, C., et al., "Microcallus Growth from Maize Protoplasts," *Planta*, 171, 58-64 (1987).

Jahne, A., et al., "Regeneration of Fertile Plants from Protoplasts Derived from Embryogenic Cell Suspensions of Barley (*Hordeum vulgare L.*)," *Plant Cell Rep.*, 10, 1–6 (1991).

Jayne, S., et al., "Anslysis of Elite Transgenic Maize Plants Produced by Microprojectile Bombardment," Program and Abstracts, Int. Soc. for Plant Mol. Biol., 3rd Int. Cong., Abstract No. 338 (Oct. 6–11, 1991).

Jefferson, R., et al., "β–Glucuronidase from *Escherichia coli* as a Gene–Fusion Marker," *Proc. Nat. Acad. Sci.* USA, 83, 8447–8451 (1986).

Jefferson, R., et al., "GUS Fusions: β–Glucuronidase as a Sensitive and Versatile Gene Fusion Marker in Higher Plants," *EMBO J.*, 6, 3901–3907 (1987).

Jefferson, R., "Assaying chimeric genes in plants: the GUS gene fusion system," *Plant Mol. Biol. Rep.*, 5, 387–405 (1987).

Jones, H., et al., "Recent Advances in Plant Electroporation," *Oxford Surveys of Plant Molecular and Cell Biol.*, 4, 347–357 (1987).

Jones, H., et al., "Transient Gene Expression in Electroporated Solanum Protoplasts," *Plant Mol. Biol.*, 13, 503–511 (1989).

Kaeppler, H. F., et al., "Silicon Carbide Fiber–Mediated DNA Delivery into Plant Cells," *Plant Cell Rep.*, 9, 415–418 (1990).

Kamo, K., et al., "Establishment and Characterization of Long–Term Embryonic Maize Callus and Cell Suspension Cultures," *Plant Sci.*, 45, 111–117 (1986).

Kamo, K, et al., "Regeneration of *Zea mays L.* from Embryogenic Callus," *Bot Gaz.*, 146, 327–334 (1985).

Kao, K. N., et al., "Nutritional Requirements for Growth of *Vicia hajastana* Cells and Protoplasts at a Very Low Population Density in Liquid Media," *Planta*, 126, 105–110 (1978).

Kartha, K., et al., "Transient Expression of Chloramphenicol Acetyl Transferase (CAT) Gene in Barley Cell Cultures and Immature Embryos Through Microprojectile Bombardment," *Plant Cell Rep.*, 8, 429–432 (1989).

Kay, R., et al., "Duplication of CaMV 35S Promoter Sequences Creates a Strong Enhancer for Plant Genes," *Science*, 236, 1299–1302 (Jun. 5, 1987).

Kirihara, J., et al., "Differential Expression of a Gene for a Methionine—Rich Storage Protein in Maize," *Mol. Gen. Genet.*, 211, 477–484 (1988).

Kirihara, J., et al., "Isolation and Sequence of a Gene Encoding a Methionine–Rich 10–kD Zein Protein from Maize," *Gene*, 71, 359–370 (1988).

Klein, T., et al., "Transfer of Foreign Genes into Intact Maize Cells with High–Velocity Microprojectiles," *Proc. Nat. Acad. Sci. USA*, 85, 4305–4309 (1988).

Klein, T. M., et al., "Factors Influencing Gene Delivery into *Zea mays* Cells by High Velocity Microprojectiles," *Bio/Tec 6, 559–563 (1988).*

Klein, T. M., et al., "High–Velocity Microprojectiles for Delivering Nucleic Acids to Living Cells," *Nature*, 327, 70–73 (1987).

Klein, T., et al., "Genetic Transformation of Maize Cell by Particle Bombardment and the Influence of Methylation on Foreign Gene Expression," In: *Gene Manipulation in Plant Improvement II*, Gustafson, J. P., (ed.), Plenum Press, NY, pp. 265–266 (1990).

Klein, T., et al., "Genetic Transformation of Maize Cells by Particle Bombardment," *Plant Physiol.*, 91, 440–444 (1989).

Klein, T., et al., "Regulation of Anthocyanin Biosynthetic Genes Introduced into Intact Maize Tissue by Microprojectiles," *Proc. Nat. Acad. Sci. USA*, 86, 6682–6685 (1989).

Kozak, M., "Compilation and Analysis of Sequence from the Translational Start Site in Eukaryotic mRNAs," *Nuc. Acids. Res.*, 12, 857–871 (1984).

Kozak, M., "Point Mutations Define a Sequence Flanking the AUG Initiator Codon that Modulates Translation by Eukaryotic Ribosomes," *Cell*, 44, 283–292 (1986).

Koziel, M. G., et al., "Field Performance of Elite Transgenic Maize Plants Expressing an Insecticidal Protein Derived from *Bacillus thuringenesis*," Bio/Technol., 11, 194–200 (1993).

Kreitlow, B., "Genetic Engineering 'Breakthrough' Disputed," Cedar Rapids Gazette (Apr. 20, 1990).

Kriz, A. L., et al., "Characterization of the Maize Globulin–2 Gene and Analysis of Two Null Alleles," *Biochemical Genetics*, 29, 241–254 (1991).

Kuhlemeier, C., et al., "Regulation of Gene Expression in Higher Plants," *Ann. Rev. Plant Physiol.*, 38, 234–239 (1987).

Langridge, et al., "Transformation of Cereals via Agrobacterium and the Pollen Pathway: A Critical Assessment," *The Plant J.*, 2, 613–638 (1992).

Laursen, C. M., et al., "Production of Fertile Transgenic Maize by Electorporation of Suspension Culture Cells," *Plant Mol. Biol.*, 24, 51–61 (1994).

Lazzeri, P., et al., "In Vitro Genetic Manipulation of Cereals and grasses," *Ad. Cell Culture*, 6, 291–293 (1988).

Lee, J. S., et al., "Gene Transfer into Intact cells of Tobacco by Electroporation," *Korea J. Gent.*, 11, 65–72 (1989).

Leemans, J., "Genetic Engineering for Fertility Control," Keystone Symposium on Crop Improvement via Biotechnology: An International Perspective, Abstract No. Y016 (Apr. 10–26, 1992).

Levitt, J. "Growth Regulators" In: *Introduction to Plant Physiology*, The C. V. Mosby Company, St. Louis, p. 241 (1969).

Li, X.–Q., et al., "GUS Expression in Rice Tissues Using Agrobacterium—Mediated Transformation," Program and Abstracts, Int. Soc. for Plant Mol. Biol., 3rd Int. Cong., Abstract No. 385 (Oct. 6–11, 1991).

Lindsey, K., et al., "Electroporation of Cells," *Physiologia Plantarum*, 79, 168–172 (1990).

Lindsey, K., et al., "The Permeability of Electroporated Cells and Protoplasts of Sugar Beet," *Planta*, 172, 346–355 (1987).

Lindsey, K., et al., "Transient Gene Expression in Electroporated Protoplasts and Intact Cells of Sugar Beet," *Plant Mol. Biol.*, 10, 43–52 (1987).

Lindsey, K. et al., "Stable Transformation of Sugarbeet Protoplasts by Electroporation," *Plant Cell Rep.*, 8, 71–74 (1989).

Looker, D., "Dekalb Claims Success in Effort to Alter Genetic Makeup of Corn," *Des Moines Register* (Apr. 19, 1990).

Lopes, M. A., et al., "Endosperm Origin, Development, and function," *The Plant Cell*, 5, 1383–1399 (1993).

Lorz, H., et al., "Advances in Tissue Culture and Progress Towards Genetic Transformation of Cereals," *Plant Breeding*, 100, 1–25 (1988).

Lu, C., et al., "Somatic Embryogenesis in *Zea mays L.*," *Theor. Appl. Genet.*," 62, 109–112 (1982).

Lu, C., et al., "Improved Efficiency of Somatic Embryogenesis and Plant Regeneration on Tissue Cultures of Maize (*Zea mays L.*)," *Theor. Appl. Genet.*, 66, 285–289 (1983).

Ludwig, S., et al., "A Regulatory Gene as a Novel Visible Marker for Maize Transformation," *Science*, 247, 449–450 (1990).

Ludwig, S., et al., "High Frequency Callus Formation from Maize Protoplasts," *Theor. Appl. Genet.*, 71, 344–350 (1985).

Ludwig, S., et al., "Lc, a Member of the Maize R Gene Family Responsible for Tissue–Specific Anthocyanin Production, Encodes a Protein Similar to Transcriptional Activators and Contains the myc–Homology Region," *Proc. Nat. Acad. Sci. USA*, 86, 7092–7096 (1989).

Ludwig, S., et al., "Maize R Gene Family: Tissue—Specific Helix–Loop–Helix Proteins," *Cell*, 62, 849–851 (1990).

Lutcke, H., et al., "Selection of AUG Initiation Codones Differs in Plants and Animals," *EMBO J.*, 6, 43–48 (1987).

Maas, C., et al., "A Highly Optimized Monocot Expression Cassette: Application for Barley Transformation and Barley Virus Research," Program and Abstracts, Int. Soc. Plant Mol. Biol., Abstract No. 386 (Oct. 6–11, (1991).

Maddock, S. E., et al., "Expression in Maize Plants of Wheat Germ Agglutinin, a Novel Source of Insect Resistance," Program and Abstracts, Int. Soc. Plant Mol. Biol., 3rd Int. Cong., Abstract no. 372 (Oct. 6–11, 1991).

Masumura, T., et al., "cDNA Cloning of an mRNA Encoding a Sulfur–Rich 10 kDa Prolamin Polypeptide in Rice Seeds," *Plant Mol. Biol.*, 12, 123–130 (1989).

McCabe et al., "Stable Transformation of Soybean (*Glycine max*) by Particle Acceleration," *Bio/Technol*, 6, 923–926 (1988).

McDaniel, C., et al., "Cell—Lineage Patterns in the Shoot Apical Meristem of the Germinating Maize Embryo," *Planta*, 175, 13–22 (1988).

Meadows, M., "Characterization of Cells and Protoplasts of the B73 Maize Cell Line," *Plant Sci. Lett.*, 28 337–348 (1982/83).

Mendel, R., et al., "Delivery of Foreign Genes to Intact Barley Cell by High–Belocity Microprojectiles," *Theor. Appl. Genet.*, 78, 31–34 (1989).

Messing, J., "Corn Storage Protein: A Molecular Genetic Model," Division of Energy BioSciences—Summaries of FY 1990 Activities, p. 70, Abstract No. 135 (1990).

Moffat, A. S., "Corn Transformed," *Science*, 249, 630 (Aug. 10, 1990).

Morkawa, et al., "Gene Transfer into Intact Plant Cells by Electroporation Through Cell Walls and Membranes," *Gene*, 41, 121 (1986).

Morocz, S. et al., "An Improved System to Obtain Fertile regenerants via Maize Protoplasts Isolated From a Highly Embryonic Suspension Culture," *Theor. Appl. Genet.*, 80, 721–726 (1990).

Morocz, S., et al., "Two Approaches to Rendering *Zea mays L.* Applicable to Tissue Culture Manipulations," Abstracts, VIIth Int. Cong. on Plant Tissue and Cell Culture, Amsterdam A1–102, Abstract No. 209, p. 190 (1990).

Murakami, T., et al., "The Bialaphos Biosynthetic Genes of *Streptomyces hygroscopicus:* Molecular Cloning and Characterization of the Gene Cluster," *Mol. Gen. Genet.*, 205, 42–50 (1986).

Murashige, T., et al., A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures," *Physiol. Plant.*, 15, 473–497 (1962).

Murphy, H. L., "New Dekalb–Pfizer Seed Chief to Harvest R & D Breakthroughs," *Crain's Business Weekly*, pp. 38–39 (1990).

Murray, E. E., et al., "Codon usage in plant geners," *Nuc. Acids Res.*, 17, 477–498 (1989).

Murry, L. E., et al., "Transgenic Corn Plants Expressing MDMV Strain B Coat Protein are Reistant to Mixed Infections of Maize Dwarf Mosaic Virus and Maize Chlorotic Mottle Virus," *Bio/Technol.*, 11, 1559–1564 (1993).

Nelson, R. S., "Virus Tolerance, Plant Growth, and Field Performance of Transgenic Tomato Plants Expressing Coat Protein from Tobacco Mosaic Virus," et al., *Bio/Technol.*, 6, 403–409 (1988).

Nelson, T., "New Horses for Monocot Gene Jockeys," *The Plant Cell*, 2, 589 (1990).

Neuffer, "Growing Maize for Genetic Purposes," Maize for Biological Research, Plant Mol. Biol. Assoc., (1988).

Odell, J., et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter," *Nature*, 313, 810–811 (1985).

Office Action dated May 30, 1989, Goldman et al., USSN 06/880,271, filed Jun. 30, 1986.

Office Action dated Mar. 8, 1990, Goldman, USSN 06/880, 271, filed Jun. 30, 1986.

Ohta, Y., "High—Efficiency Genetic Transformation of Maize by a Mixture of pollen and Exogenous DNA," *Proc. Nat. Acad. Sci. USA*, 83, 715–719 (1986).

Okta, Y., et al., "Gene Manifestation of Exogenuous DNA Applied to Self—Propagating Stigma (Gene Action Revealed in the M_1 and M_2 Generations from Self–Pollination Applying Exogenous DNA)," *Jap. J. Breed.*, 30 184–185 (1980).

Omirullen, S., et al., "Activity of a Chimeric Promoter with the Doubled CaMV 35S Enhancer in Protoplast–Derived Cells and Transgenic Plants in Maize," *Plant Mol. Biol.*, 21, 415–428 (1993).

Ozias–Akins, P., et al., "In vitro regeneration and genetic manipulation of grasses," *Physiol. Plant.*, 73, 565–569 (1988).

Ozias–Akins, P., et al., "Progress and Limitations in the Culture of Cereal Protoplasts," *Trends in Biotechnol*, 2, 119–123 (1984).

Park, S. H., et al., "Selection of Maize Transformants from Shoot Apex cultures Cocultivated with Agrobacterium Containing the Bar Gene," *In Vitro Cell. Develop. Biol.*, 29A, p. 85A, Abstract No. P–1102 (1993).

Parker, W. B., et al., "Selection and Characterization of Sethoxydim—Tolerant Maize Tissue Cultures," *Plant Physiol.*, 92, 1220–1225 (1990).

Pederson, K., et al., "Sequence Analysis and Characterization of a Maize Gene Encoding a High–Sulfur Zein Protein of M_r 15,000," *J. Biol. Chem.*, 261, 6279–6284 (1986).

Perl, A., et al., "Bacterial Dihydrodipicolinate Synthase and Desensitized Aspartate Kinase: Two Novel Selectable Markers for Plant Transformation," *Bio/Technol.*, 11, 715–718 (1993).

Perlack, F. J., et al., "Modification of the Coding Sequence Enhances Plant Expression of Insect Control Protein Genes," *Proc. Nat. Acad. Sci. USA*, 88, 3324–3328 (1991).

Phillips, R. L., et al., "Elevated Protein—Bound Methionine in Seeds of a Maize Line Resistant to Lysine Plus Threonine," *Cerial Chem.*, 62, 213–218 (1985).

Phillips, R. L., et al., "Cell/Tissue Culture and In Vitro Manipulation," In: *Corn and Corn Improvement,* 3rd edition, Sprague, G. F., et al., (eds.), Agronomy Soc. Amer., pp. 345–387 (1988).

Pioneer HiBred International, Inc., *Application Under 7 CFR 340,* Release of Genetically Engineered Corn Plants, Permit No. 92–174–02, NO CBI, p. 8 (Nov. 3, 1992).

Pioneer HiBred International, Inc., *Application Under 7 CFR 340,* Release of Genetically Engineered Corn Plants, Permit No. 92–330–01, CBI–Deleted, p. 13 (Apr. 13, 1993).

Philips, R. L., et al., "Elevated Protein—Bound Methionine in Seeds of a Maize Line Resistant to Lysine plus Threonine," *Cereal Chem.,* 62, 213–218 (1985).

Pioneer's Application for Release in the *Environment Under 7CFR 340,* Corn Plants Genetically Engineered to Express Wheat Germ Agglutinin (WGA) Genes, in Order to Confer Resistance to the Europeans Corn Borer (*Ostrinia nubilalis*) and Tolerance to Glufosinate Herbicides, 92–022–02, No CBI Copy, p. 11 (May 4, 1992).

Pochlman, J. "Breeding Corn (Maize)," In: *Breeding Field Crops,* 3rd edition, AVI Publishing Co., Westport CN, pp. 452 (1986).

Proehlman, J. Breeding Corn (Maize), "In: *Breeding Field Crops,* 3rd edition, AVI Publishing Co., Westport CN, pp. 469–471, 477–481 (1986).

Potrykus, I., et al., "Callus Formation from Cell Culture Protoplasts of Corn (*Zea mays L.*)," *Theor. Appl. Genet.,* 54, 209–214 (1979).

Potrykus, I., "Gene Transfer to Cereals: An Assessment," *Bio/Technol.,* 8, 535–542 (Jun. 1990).

Potrykus, I., "Gene Transfer to Cereals: An Assessment," *Trends Biotechnol,* 7, 269–273 (Oct. 1989).

Potrykus, I., "Gene Transfer to Plants: Assessment and Perspectives," *Physiol. Plant.,* 79, 125–134 (1990).

Potrykus, I., et al., "Callus formation from stem protoplasts of corn (*Zea mays L.*)" *Mol. Gen. Genet.,* 156, 347–350 (1977).

Potter, et al., "Enhancer—Dependent Expression of Human K Immunoglobulin Genes Introduced into Mouse Pre–B Lymphocytes by Electroporation," *Proc. Nat. Acad. Sci. USA,* 81, 7161 (1984).

Prioli, L. M., et al., "Plant Regeneraton and Recovery of Fertile Plants from Protoplasts of Maize (*Zea mays L.*)," *Bio/Technol.* 7, 589–594 (Jun. 1989).

Puite, K. J., et al., "Electrofusion, a Simple and Reproducible Technique in Somatic Hybridization of *Nicotiana plumbaginifolia* mutants," *Plant Cell Rep.,* 4, 274–276 (1985).

Rasmusen, J. L., et al., "Biolistic Transformation of Tobacco and Maize Suspension Cells Using Bacterial Cells as Microprojectiles," *Plant Cell Rep.* 13, 212–217 (1994).

Rhodes, C. A., et al., "Genetically Transformed Maize Plants from Protoplasts," *Science,* 240, 204–207 (Apr. 8, 1988).

Rhodes, C. A., et al., "Plant Regeneration from Protoplasts Isolated from Embryogenic Maize Cell Cultures," *Bio/Technol.,* 6, 56–60 (Jan. 1988).

Rhodes, C. A., "Corn: From Protoplasts to Fertile Plants," *Bio/Technol.,* 7, 548 (Jun. 1989).

Richaud, F., et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli dapA* Gene," *Biol. Abstracts,* 82, p. AB–391, Abstract No. 3396 (1986).

Richaud, F., et al., "Chromosomal Location and Nucleotide Sequence of the *Escherichia coli dapA* Gene," *J. Bacteriol,* 166, 297–300 (1986).

Robbins–Roth et al., "They Make it Happen in Biotech," *Bioworld,* pp. 30–36 (Nov./Dec. 1990).

Robertson, D. S., "Loss of Mu Mutator Activity when Active Mu Systems are Transferred to Inbred Lines," *Maize Genetics Coop. Newsletter,* 60, 10 (1986).

Ross, M. C., et al., "Transient and Stable Transgenic Cells and Calli of Tobacco and Maize Following Microprojectile Bombardment," *J. Cell. Biochem.,* 13D, p. 268, Abstract No. M149 (1989).

Sahi, S. V., et al., "Metabolites in Maize Which Affect Virulence Induction in *Agrabacterium tumefaciens," Plant Physiol, Supplement,* p. 86, Abstract No. 514, (1989).

Sanford, J. C., "Biolistic Plant Transformation," *Physiol. Plant.,* 79, 206–209 (1990).

Sanford, J. C., "The Biolistic Process," *Trends Biotechnol.,* 6, 299–302 (1988).

Sanford, J. C., et al., "Attempted Pollen—Mediated Plant Transformation Employing Genomic Donor DNA," *Theor. Appl. Genet.,* 69, 571–574 (1985).

Sanford, J. C., et al., "Delivery of Substances into Cells and Tissues Using a Particle Bombardment Process," *Particulate Sci. Technol.,* 5, 27–37 (1987).

Sass, "Morphology: Development of the Caryopsis" In: *Corn and Corn Improvement,* 2nd edition, Sprauge, G. F., (ed.), American Soc. Agronomy, p. 89, 98 (1977).

Schmidt, A., et al., "Media and environmental effects of phenolics production from tobacco cell cultures," *chem. Abstracts,* 110, p. 514, Abstract No. 230156z (1989).

Shen, W,–H., et al., "Excision of a Transposible Element form a Viral Vector Introduced into Maize Plants by Agroinfection," *The Plant J.,* 2, 35–42 (1992).

Shen, W.–H., et al., "Amplification and expression of the β–glucuronidase gene in maize plants by vectors based on maize streak virus," *The Plant Journal,* 5, 227–236 (1994).

Shigekawa, K., et al., "Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells," *Bio Techniques,* 6, 742–751 (1988).

Shillito, R. D., et al., "High Efficiency Direct Gene Transfer to Plants," *Bio/Technol.,* 3, 1099 (1985).

Shillito, R. D., et al., "Regeneration of Fertile Plants From Protoplasts of Elite Inbred Maize," *Bio/Technol.,* 7, 581–587 (Jun. 1989).

Shimamoto, K., et al., "Fertile Transgenic Rice Plants Regenerated from Transformed Protoplasts," *Nature,* 338, 274–278 (1989).

Shotwell, M. A., et al., "The Biochemistry of Plants—A Comprehensive Treatise," In: *The Biochemistry of Plants,* vol. 15, Marcus, A., (ed.), Academic Press, Inc., San Diego, pp. 297–345 (1989).

Smith, R., et al., "Shoot apex explant for transformation," *Plant Physiol.,* 86, p. 108, Abstract No. 646 (1988).

Soberon, X., et al., "Construction and Characterization of New Cloning Vehicles, IV. Deletion Derivatives of pBR322 and pBR325," *Gene,* 9, 287–305 (1980).

Songstad, D. D., et al., "Transient Expression of GUS and Anthocyanin Constructs in Intact Maize Immature Embryos Following Electroporation," *Plant Cell Tissue and Organ Culture,* 33, 195–201 (1993).

Spencer, T. M. et al., "Fertile Transgenic Maize," Abstracts, 7th Annual Meeting, Mid Atlantic Plant Mol. Biol. Soc. p. 30 (1990).

Spencer et al., "Bialaphos Selection of Stable Transformations from Maize Cell Culture," *Theor. Appl. Genet.,* 79, 625–631 (May 1990).

Spencer, T. M., et al., "Segregation of Transgenes in Maize," *Plant Mol. Biol.,* 18, 201–210 (1992).

Spencer, T. M., et al., "Selection of Stable Transformants from Maize Suspension Cultures using the Herbicide Bialaphos," Poster presentation, FASEB Plant Gene Expression Conference, Copper Mountain, Colorado (Aug. 8, 1989).

Sprauge et al., "Corn Breeding," In: *Corn and Corn Improvement,* Sprauge, G. F., (ed.), American Society of Agronomy, Inc, Madison, Wi, pp. 305, 320–323 (1977).

Steimel, D., "Corn Breeders Stalk Perfect Hybrid," *Rockford Register Star.* (Aug. 6, 1990).

Steimel, D., "New Gun Will Custom–Design Corn: Breeding Technique Expected by End of '90's Will Let Crop Grow Without Pesticides or Much Water," (Apr. 1990).

Sugiyama, M., et al., "Use of the Tyrosinase Gene from Streptomyces to Probe Promoter Sequences for *Escherichia coli,*" *Plasmid,* 23, 237–241 (1990).

Suttie, J., et al., "Use of Different Selection Agents to Produce Maize Transformants of an Elite Genotype Using Microprojectile Bombardment," Program and Abstracts, Int. Soc. Plant Mol. Biol., 3rd Int. Cong., Abstract No. 426 (Oct. 6–11, 1991).

Tarczynski, M. C., et al., "Expression of a Bacterial mtlD Gene in Transgenic Tobacco Leads to Production and Accumulation of Mannitol," *Proc. Nat. Acad. Sci. USA,* 89, 2600–2604 (1992).

Tarczynski, m. C., et al., "Stress Protection of Transgenic Tobacco by Production of the Osmolyte Mannitol," *Science,* 259, 508–510 (1993).

Thompson, C., et al., "Characterization of the Herbicide–Resistance Gene bar from *Streptomyces hygroscopicus,*" *EMBO J.,* 6, 2519–2523 (1987).

Tomes, D. "Status of Corn Transformation," 26th Annual Corn Breeders School, Meeting Proceedings, U. Illinois, pp. 7–8 (Feb. 26–27, 1990).

Tomes, D. T., et al., "Transgenic Tobacco Plants and their Progeny Derived by Microprojectile Bombardment of Tobacco Leaves," *Plant Mol. Biol.,* 14, 261–261–268 (Feb. 1990).

Twell, D., et al., "Transient Expression of Chimeric Genes Delivered into Pollen by Microprojectile Bombardment," *Plant Physiol.,* 91, 1271–1274 (1989).

Ulian, E., et al., "Transformation of Plants via the Shoot Apex," *In Vitro Cell. Dev. Biol.,* 9, 951–954 (1988).

Usami, S., et al., "Absence in Monocotyledonous Plants of the Diffusible Plant Factors including T–DNA Circularization and vir Gene Expression in Agrobacterium," *Mol. Gen. Genet.,* 209, 221–226 (1987).

Vain, P., et al., "Osmotic Pretreatment Enhances Particle Bombardment–Mediated Transient and Stable Transformation of Maize," *Plant Cell Rep.,* 12, 84–88 (1993).

Vasil, I. K., "Transgenic Cereals Becoming a Reality," *Bio/Technol.,* 8, 797 (Sep. 1990).

Vasil, I. K., et al., "Culture of Protoplasts Isolated from Embryogenic Cell Suspension Cultures of Sugarcane and Maize," *IAPTC Abstracts,* p. 443 (1986).

Vasil, V., et al., "Isolation and Maintenance of Embryogenic Cell Suspension Cultures of Gramineae," In: *Cell Culture and Somatic Cell Genetics of Plants,* vol. I, Academic Press, pp. 152–158 (1984).

Vasil, V., et al., "Plant Regeneration from Friable Embryonic Callus and Cell Suspension Cultures of *Zea mays L.,*" *J. Plant Physiol.,* 124, 399–408 (1986).

Walbot, V., et al., "Molecular genetics of corn," In: *Corn and Corn Improvements,* 3rd edition, Sprauge, G. F., et al., (eds.), American Soc. Agronomy, Madison, WI, pp. 389–430 (1988).

Waldron, C., et al., "Resistance to Hygromycin B," *Plant Mol. Biol.,* 5, 103–108 (1985).

Walters, D. A., et al., "Transformation and Inheritance of Hygromycin Phosphotransferase Gene in Maize Plants," *Plant Molecular Biol.,* 18, 189–200 (1992).

Wan, Y., et al., "Generation of Large Numbers of Independently Transformed Fertile Barley Plants," *Plant Physiol.,* 104, 37–48 (1994).

Wan, Y., et al., "Maize Transformation and Regeneration of Transgenic Plants by Microprojectile Bombardment of Type I Callus," Abstracts, 35th Annual Maize Genetics Conference, p. 5 (Mar. 18–21, 1993).

Wang, Y., et al., "Characterization of cis–Acting Elements Regulating Transcription from the Promoter of a Constitutively Active Rice Actin Gene," *Mol. Cell. Biol.,* 12, 3399–3406 (1992).

Wang, Y., et al., "Transient Expression of Foreign Genes in Rice, Wheat and Soybean Cells Following Particle Bombardment," *Plant Mol. Biol.,* 11, 433–439 (1988).

Weising, K., et al., "Foreign Genes in Plants: Transfer, Structure, Expression and Applications," *Ann. Rev. Genet.,* 22, 421–478 (1988).

White, J., et al., "A Cassette Containing the bar Gene of *Streptomyces hygroscopicus:* a Selectable Marker for Plant Transformation," *Nuc. Acid. Res.,* 18, 1062 (1989).

Whiteley, H. R., et al., "The Molecular Biology of Parasporal Crystal Body Formation in *Bacillus thuringiensis, Ann. Rev. Microbiol.,* 40, 549–576 (1986).

Wong, E. Y., et al., "*Arabidopsis thaliana* Small Subunit Leader and Transit Peptide Enhance the Expression of *Bacillus thuringiensis* Proteins in Transgenic Plants," *Plant Mol. Biol.,* 20, 81–93 (1992).

Yang, H., et al. "Production of Kanamycin Resistant Rice Tissues Following DNA Uptake into Protoplasts," *Plant Cell Rep.* 7, 421 (1988).

Yanisch–Perron, C., et al., "Improved M13 Phage Vectors and Host Strains: Nucleotide Sequences of the M13mp18 and puC19 Vectors," *Gene,* 33, 103–119 (1985).

Yugari, Y., et al., "Coordinated End—Product Inhibition in Lysine Synthesis in *Escherichia coli,*" *Biochem. Biophys. Acta.* 62, 612–614 (1962).

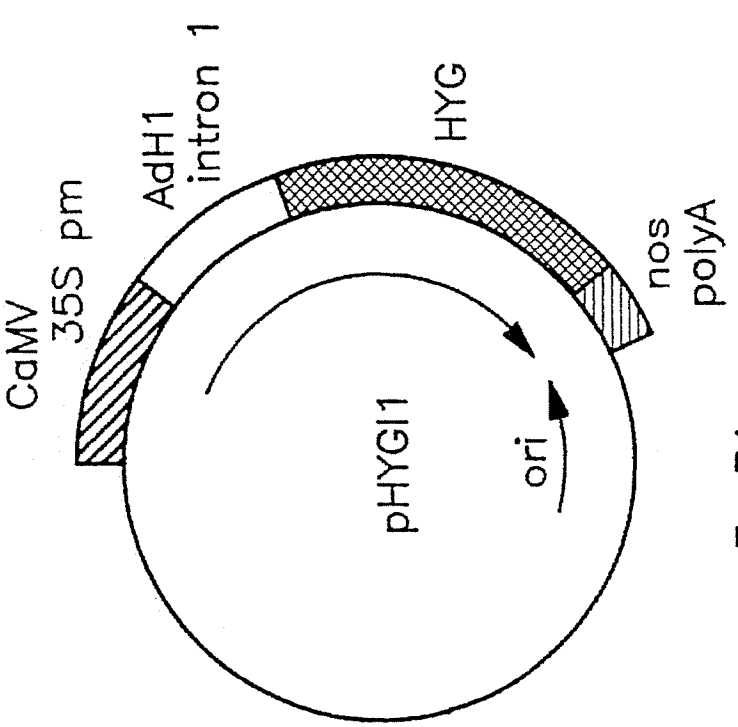

CaMV
35S pm
AdH1
intron 1
HYG
nos
polyA
pHYG11
ori

FIG. 1A

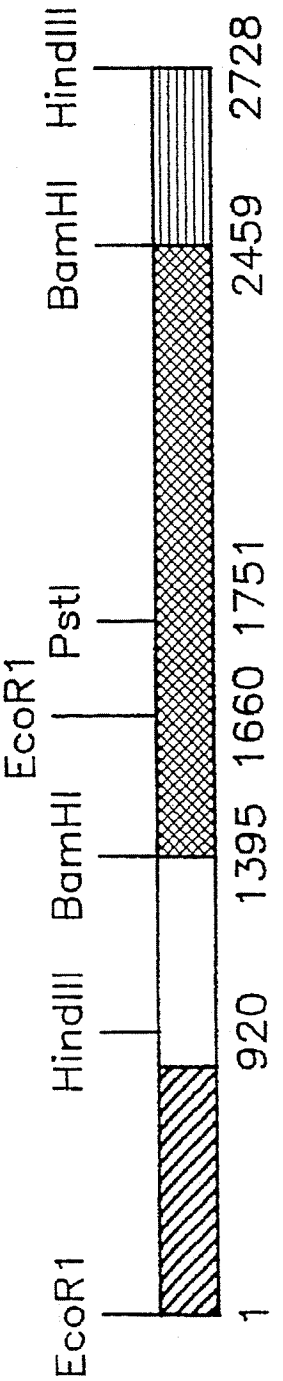

EcoR1
HindIII
BamHI
PstI
EcoR1
BamHI
HindIII
1
920
1395
1660
1751
2459
2728

FIG. 1B

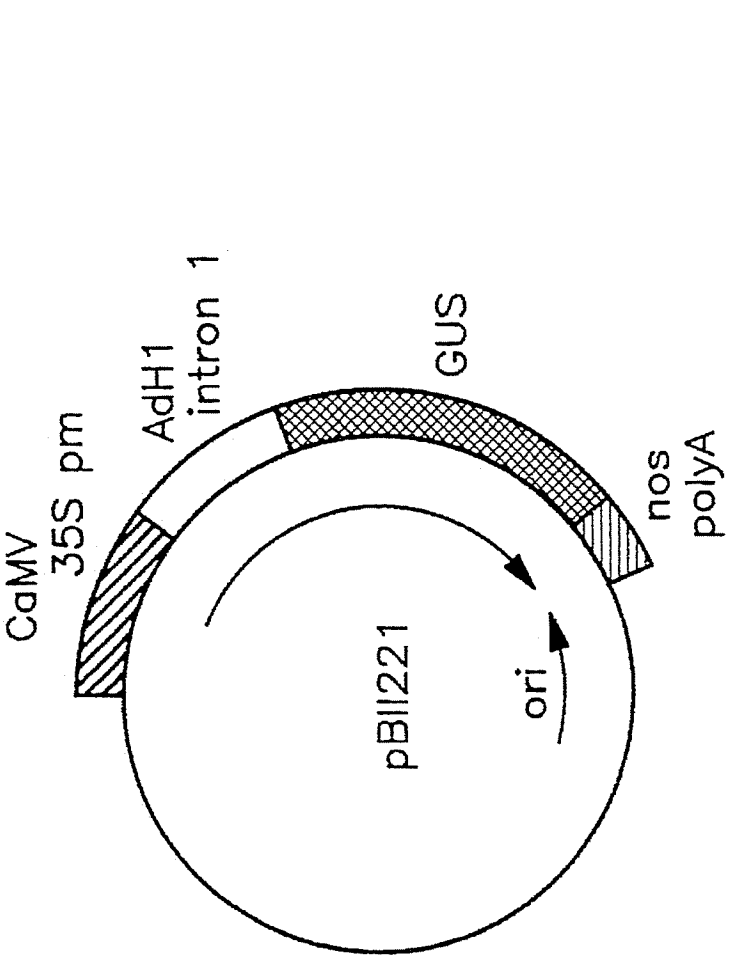

CaMV 35S pm
AdH1 intron 1
GUS
nos polyA
pBII221
ori

FIG. 2A

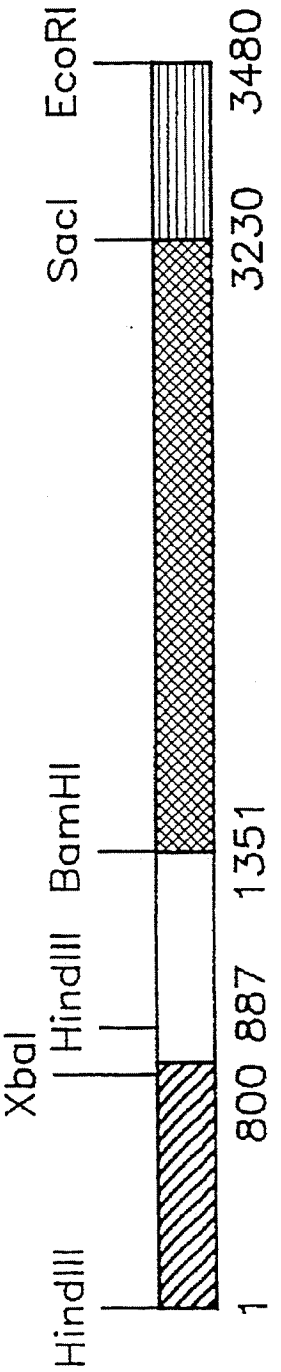

EcoRI
3480
SacI
3230
BamHI
1351
XbaI
HindIII
887
800
HindIII
1

FIG. 2B

FIG. 3

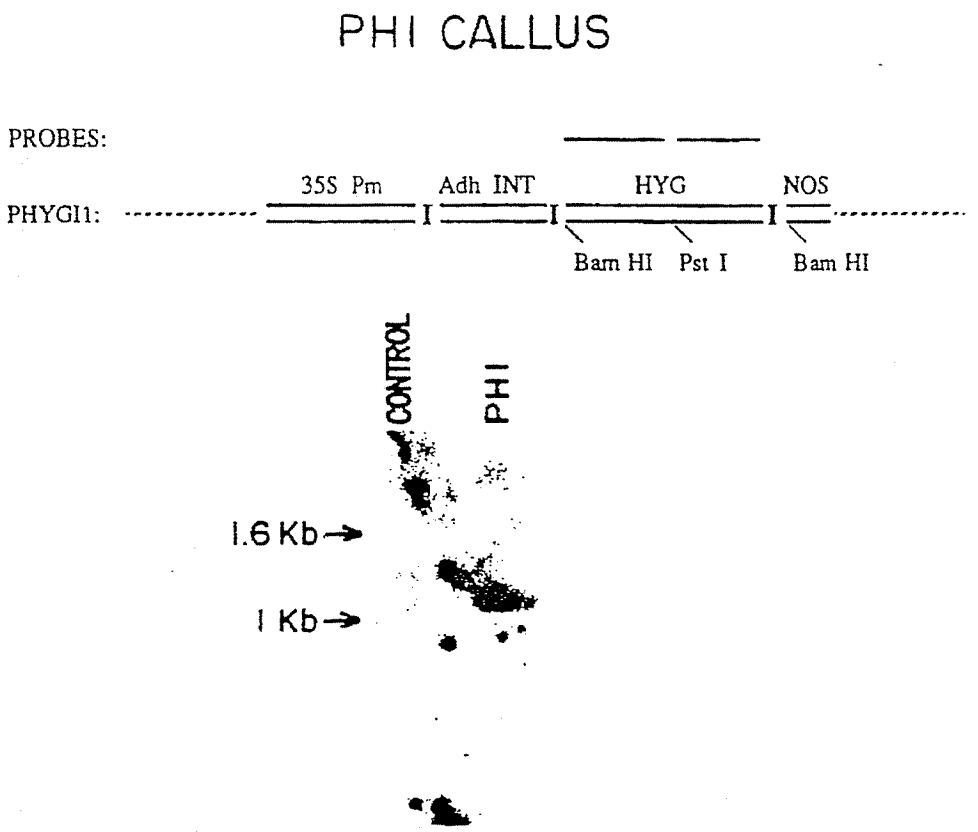

PHI CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI
CONTROL
PHI
1.6 Kb
1 Kb

FIG. 4

PHI R_o PLANTS

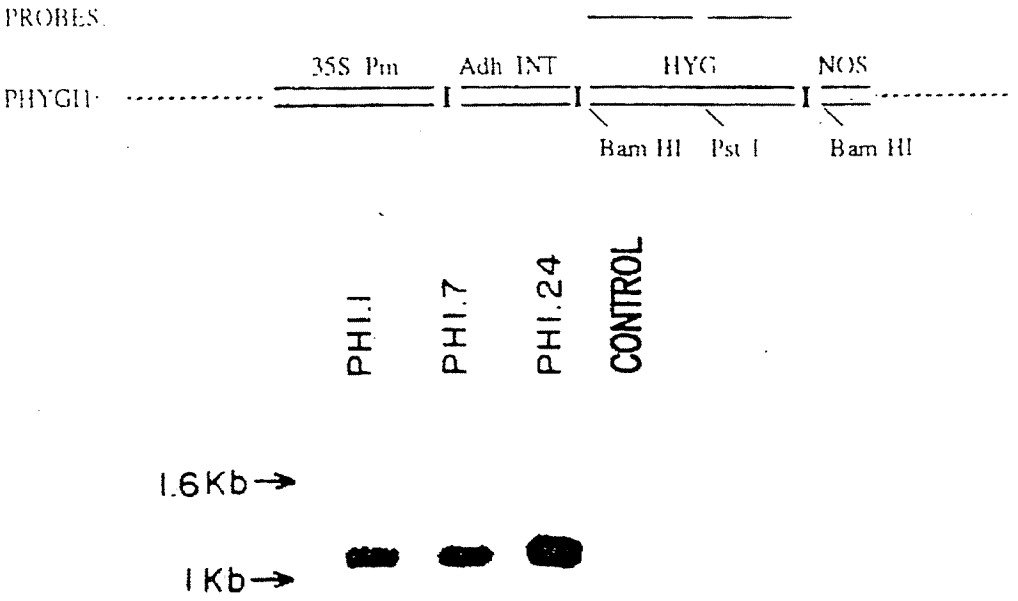


FIG. 5

PHI R_1 GENERATION

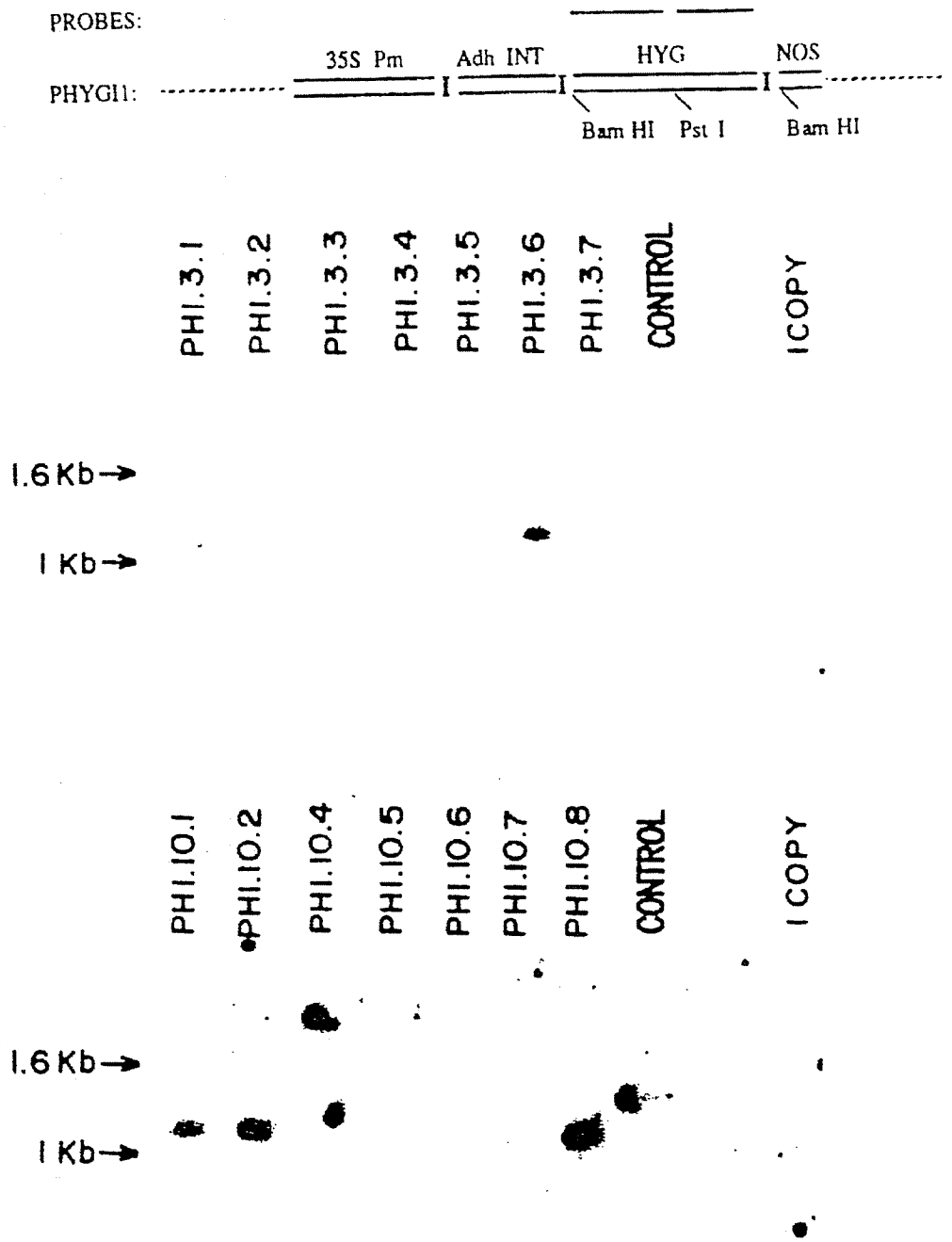
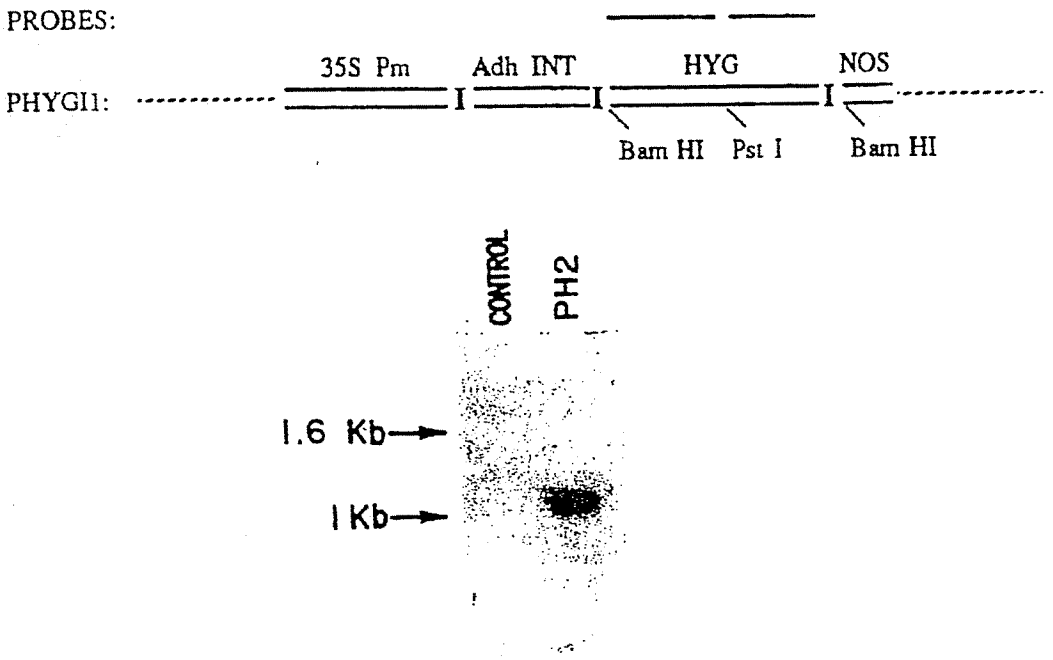


FIG. 6

PH2 CALLUS



FERTILE GLYPHOSATE-RESISTANT TRANSGENIC CORN PLANTS

CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation of application Ser. No. 07/508,045, filed Apr. 11, 1990, now U.S. Pat. No. 5,484,956, which is a continuation-in-part of U.S. patent application Ser. No. 07/467,983, filed Jan. 22, 1990, now abandoned.

FIELD OF THE INVENTION

This invention relates to fertile transgenic plants of the species *Zea mays* (oftentimes referred to herein as maize or corn). The invention further relates to producing transgenic plants via particle bombardment and subsequent selection techniques which have been found to produce fertile transgenic plants.

BACKGROUND OF THE INVENTION

Genetic engineering of plants, which entails the isolation and manipulation of genetic material (usually in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells, offers considerable promise to modern agriculture and plant breeding. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals, chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques. Once a gene has been identified, cloned, and engineered, it is still necessary to introduce it into a plant of interest in such a manner that the resulting plant is both fertile and capable of passing the gene on to its progeny.

A variety of methods have been developed and are currently available for the transformation of various plants and plant cells with DNA. Generally, these plants have been dicotyledonous, and some success has been reported with certain of the monocotyledonous cereals. However, some species have heretofore proven untransformable by any method. Thus, previous to this discovery, no technology had been developed which would permit the production of stably transformed *Zea mays* plants in which the transforming DNA is heritable thereof. This failure in the art is well documented in the literature and has been discussed in a number of recent reviews (Potrykus, 1989; Weising et al., 1988; Cocking et al., 1987).

European Patent Publications 270,356 (McCabe et al.) and 275,069 (Arntzen et al.) describe the introduction of DNA into maize pollen followed by pollination of maize ears and formation of seeds. The plants germinated from these seeds are alleged to contain the introduced DNA, but there is no suggestion that the introduced DNA was heritable, as has been accomplished in the present invention. Only if the DNA introduced into the corn is heritable can the corn be used in breeding programs as required for successful commercialization of transgenic corn.

Graves et al. (1986) claim Agrobacterium-mediated transformation of *Zea mays* seedlings. The evidence was based upon assays known to be unreliable.

Despite extensive efforts to produce fertile transformed corn plants which transmit the transforming DNA to progeny, there have been no reported successes. Many previous failures have been based upon gene transfer to maize protoplasts, oftentimes derived from callus, liquid suspension culture cells, or other maize cells using a variety of transformation techniques. Although several of the techniques have resulted in successful transformation of corn cells, the resulting cells either could not be regenerated into corn plants or the corn plants produced were sterile (Rhodes et al. 1988) or, in some cases, it even turned out that the plants were, in fact, not transformed. Thus, while maize protoplasts and some other cells have previously been transformed, the resulting transformants could not be regenerated into fertile transgenic plants.

On the other hand, it has been known that at least certain corn callus can be regenerated to form mature plants in a rather straightforward fashion and that the resulting plants are often fertile. However, no stable transformation of maize callus was ever achieved, i.e., there were no techniques developed which would permit a successful stable transformation of a regenerable callus. An example of a maize callus transformation technique which has been tried is the use of Agrobacterium-mediated transfer.

The art was thus faced with a dilemma. While it was known that corn protoplast and suspension culture cells could be transformed, no techniques were available which would regenerate the transformed protoplast into a fertile plant. While it was known that corn callus could be regenerated into a fertile plant, there were no techniques known which could transform the callus, particularly while not destroying the ability of the callus both to regenerate and to form fertile plants.

Recently, a new transformation technique has been created based upon the bombardment of intact cells and tissues with DNA-coated microprojectiles. The technique, disclosed in Sanford et al. (1987) as well as in EPO Patent Publication 331,855 of J. C. Sanford et al. based upon U.S. Ser. No. 07/161,807, filed Feb. 29, 1988, has been shown effective at producing transient gene expression in some plant cells and tissues including those from onion, maize (Klein et al. 1988a), tobacco, rice, wheat, and soybean, and stable expression has been obtained in tobacco and soybeans. In fact, stable expression has been obtained by bombardment of suspension cultures of *Zea mays* Black Mexican Sweet (Klein et al. 1989) which cultures are, however, non-regenerable suspension culture cells, not the callus culture cells used in the process of the present invention.

No protocols have been published describing the introduction of DNA by a bombardment technique into cultures of regenerable maize cells of any type. No stable expression of a gene has been reported by means of bombardment of corn callus followed by regeneration of fertile plants and no regenerable fertile corn has resulted from DNA-coated microprojectile bombardment of the suspension cultures. Thus, the art has failed to produce fertile transformed corn plants heretofore.

A further stumbling block to the successful production of fertile transgenic maize plants has been in selecting those few transformants in such a manner that neither the regeneration capacity nor the fertility of the regenerated transformant are destroyed. Due to the generally low level of transformants produced by a transformation technique, the need for selection of the transformants is self-evident. However, selection generally entails the use of some toxic agent, e.g., herbicide or antibiotic, which may be detrimental to either the regenerability or the resultant plant fertility.

It is thus an object of the present invention to produce fertile, stably transgenic, *Zea mays* plants and seeds which

transmit the introduced gene to progeny. It is a further object to produce such stably transgenic plants and seeds by a particle bombardment and a selection process which results in a high level of viability for at least a few transformed cells. It is a further object to produce fertile stably transgenic plants of other graminaceous cereals besides maize.

REFERENCES CITED

The references listed below are incorporated by reference herein.

Armstrong, C. L., et al. (1985) Planta 164:207–214

Callis, J., et al. (1987) Genes & Develop 1:1183–1200

Chilton & Barnes (1983) Nuc Acids Res 11:364–385

Chu, C. C., et al. (1975) Sci Sin (Peking) 18:659–668

Cocking, F., et al. (1987) Science 236:1259–1262

DeWet et al. (1985) Proc Natl Sci USA 82:7870–7873

Freeling, J. C., et al. (1976) Maydica XXI:97–112

Graves, A., et al. (1986) Plant Mol Biol 7:43–50

Green, C., et al. (1975) Crop Sci 15:417–421

Green, C., et al. (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 367–372

Gritz, L., et al. (1983) Gene 25:179–188

Guilley, H., et al. (1982) Cell 30:763–773

Hallauer, A. R., et al. (1988) Corn and Corn Improvement, 3rd ed., Agronomy Society of America, pp 469–564

Jefferson, R., et al. (1987) EMBO J 6:3901–3907

Kamo, K., et al. (1985) Bot Gaz 146:327–334

Klein, T., et al. (1989) Plant Physiol 91:440–444

Klein, T., et al. (1988a) Proc Natl Acad Sci USA 85:4305–9

Klein, T., et al. (1988b) Bio/Technology 6:559–563

Lu, C., et al. (1982) Theor Appl Genet 62:109–112

McCabe, D., et al. (1988) Bio/Technology 6:923–926

Murashige, T., et al. (1962) Physiol Plant 15:473–497

Neuffer, M., (1982) Maize for Biological Research, Plant Mol Biol Assoc, pp 19–30

Phillips, R., et al. (1988) Corn and Corn Improvement, 3rd ed., Agronomy Society of America, pp 345–387

Potrykus, I. (1989) Trends in Biotechnology 7:269–273

Rhodes, C. A., et al. (1988) Science 240:204–7

Sambrook, J., et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press

Sanford, J., et al. (1987) J Part Sci & Techn 5:27–37

Weising, K., et al., (1988) Ann Rev of Genetics 22:421–478

Yanisch-Perron, L., et al. (1985) Gene 33:109–119

SUMMARY OF THE INVENTION

The present invention relates to fertile transgenic *Zea mays* plants containing heterologous DNA, preferably chromosomally integrated heterologous DNA, which is heritable by progeny thereof.

The invention further relates to all products derived from transgenic *Zea mays* plants, plant cells, plant parts, and seeds.

The invention further relates to transgenic *Zea mays* seeds stably containing heterologous DNA and progeny which have inherited the heterologous DNA. The invention further relates to the breeding of transgenic plants and the subsequent incorporation of heterologous DNA into any *Zea mays* plant or line.

The invention further relates to a process for producing fertile transgenic *Zea mays* plants containing heterologous DNA. The process is based upon microprojectile bombardment, selection, plant regeneration, and coventional backcrossing techniques.

The invention further relates to a process for producing fertile transformed plants of graminaceous plants other than *Zea mays* which have not been reliably transformed by traditional methods such as electroporation, Agrobacterium, injection, and previous ballistic techniques.

The invention further relates to regenerated fertile mature maize plants obtained from transformed embryogenic tissue, transgenic seeds produced therefrom, and R1 and subsequent generations.

In preferred embodiments, this invention produces the fertile transgenic plants by means of a DNA-coated microprojectile bombardment of clumps of friable embryogenic callus, followed by a controlled regimen for selection of the transformed callus lines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows a map of plasmid vector pHYGI1 utilized in Example I. FIG. 1B shows the relevant part of pHYGI1 encompassing the HPT coding sequence and associated regulatory elements. The base pair numbers start from the 5' nucleotide in the recognition sequence for the indicated restriction enzymes, beginning with the EcoRI site at the 5' end of the CaMV 35S promoter.

FIG. 2 shows a map of plasmid vector pBII221 utilized in Example I.

FIG. 3 is a Southern blot of DNA isolated from the PH1 callus line and an untransformed control callus line.

FIG. 4 is a Southern blot of leaf DNA isolated from Ro plants regenerated from PH1 and untransformed callus.

FIG. 5 is a Southern blot of leaf DNA isolated from R1 progeny of PH1 Ro plants and untransformed Ro plants.

FIG. 6 is a Southern blot of DNA isolated from the PH2 callus line and an untransformed control callus line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the production of fertile transgenic plants and seeds of the species *Zea mays* and to the plants, plant tissues, and seeds derived from such transgenic plants, as well as the subsequent progeny and products derived therefrom. The transgenic plants produced herein include all plants of this species, including field corn, popcorn, sweet corn, flint corn and dent corn.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered beneficially by the presence of heterologous DNA that was introduced into the genotype by a process of genetic engineering, or which was initially introduced into the genotype of a parent plant by such a process and is subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions. As used herein, "genotype" refers to the sum total of genetic material within a cell, either chromosomally, or extrachromosomally borne. Therefore, the term "transgenic" as used herein does not encompass the alteration of the genotype of *Zea mays* by conventional plant breeding methods or by naturally occurring

events such as random cross-fertilization or spontaneous mutation.

By "heritable" is meant that the DNA is capable of transmission through a complete sexual cycle of a plant, i.e., it is passed from one plant through its gametes to its progeny plants in the same manner as occurs in normal corn.

The transgenic plants of this invention may be produced by (i) establishing a regenerable cell culture, preferably a friable embryogenic callus from the plant to be transformed, (ii) transforming said cell culture by a microprojectile bombardment technique, (iii) controllably identifying or selecting transformed cells, and (iv) regenerating fertile transgenic plants from the transformed cells. Some of the plants of this invention may be produced from the transgenic seed produced from the fertile transgenic plants using conventional crossbreeding techniques to develop transgenic elite lines and varieties, or commercial hybrid seed containing heterologous DNA.

I. Plant Lines and Tissue Cultures

The cells which have been found particularly useful to produce the fertile transgenic maize plants herein are those callus cells which are regenerable, both before and after undergoing a selection regimen as detailed further below. Generally, these cells will be derived from meristematic tissue which contain cells which have not yet terminally differentiated. Such tissue in graminaceous cereals in general and in maize, in particular, comprise tissues found in juvenile leaf basal regions, immature tassels, immature embryos, and coleoptilar nodes. Preferably, immature embryos are used. Methods of preparing and maintaining callus from such tissue and plant types are well known in the art and details on so doing are available in the literature, c.f. Phillips et al. (1988), the disclosure of which is hereby incorporated by reference.

The specific callus used must be able to regenerate into a fertile plant. The specific regeneration capacity of particular callus is important to the success of the bombardment/selection process used herein because during and following selection, regeneration capacity may decrease significantly. It is therefore important to start with cultures that have as high a degree of regeneration capacity as possible. Callus which is more than about 3 months and up to about 36 months of age has been found to have a sufficiently high level of regenerability and thus is preferred. The regenerative capacity of a particular culture may be readily determined by transferring samples thereof to regeneration medium and monitoring the formation of shoots, roots, and plantlets. The relative number of plantlets arising per petri dish or per gram fresh weight of tissue may be used as a rough quantitative estimate of regeneration capacity. Generally, a culture which will produce at least one plant per gram of callus tissue is preferred.

While maize callus cultures can be initiated from a number of different plant tissues, the cultures useful herein are preferably derived from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1–3 mm in length. This length generally occurs about 9–14 days after pollination. Under aseptic conditions, the embryos are placed on conventional solid media with the embryo axis down (scutellum up). Callus tissue appears from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" it is meant that the tissue is easily dispersed without causing injury to the cells. Tissue with this morphology is then transferred to fresh media and subcultured on a routine basis about every two weeks.

The callus initiation media is solid because callus cannot be readily initiated in liquid medium. In preferred embodiments, the initiation/maintenance media is typically based on the N6 salts of Chu et al. (1975) as described in Armstrong et al. (1985) or the MS salts of Murashige et al. (1962). The basal medium is supplemented with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth. The cultures are generally maintained in the dark, though low light levels may also be used. The level of synthetic hormone 2,4-D, necessary for maintenance and propagation, should be generally about 0.3 to 3.0 mg/l.

Although successful transformation and regeneration has been accomplished herein with friable embryogenic callus, this is not meant to imply that other transformable regenerable cells, tissue, or organs cannot be employed to produce the fertile transgenic plants of this invention. The only actual requirement for the cells which are transformed is that after transformation they must be capable of regeneration of a plant containing the heterologous DNA following the particular selection or screening procedure actually used.

II. DNA Used for Transformation

As used herein, the term "heterologous DNA" refers to a DNA segment that has been derived or isolated from one genotype, preferably amplified and/or chemically altered, and later introduced into a *Zea mays* genotype that may be the same *Zea mays* genotype from which the DNA was first isolated or derived. "Heterologous DNA" also includes completely synthetic DNA, and DNA derived from introduced RNA. Generally, the heterologous DNA is not originally resident in the *Zea mays* genotype which is the recipient of the DNA, but it is within the scope of the invention to isolate a gene from a given *Zea mays* genotype, and to subsequently introduce multiple copies of the gene into the same genotype, e.g., to enhance production of an amino acid.

Therefore, "heterologous DNA" is used herein to include synthetic, semi-synthetic, or biologically derived DNA which is introduced into the *Zea mays* genotype, and retained by the transformed *Zea mays* genotype. The DNA includes but is not limited to, non-plant genes such as those from bacteria, yeasts, animals or viruses; modified genes, portions of genes, chimeric genes, as well as genes from the same or different *Zea mays* genotype.

The heterologous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of a plasmid and contains coding regions of beneficial heterologous DNA with flanking regulatory sequences which promote the expression of the heterologous DNA present in the resultant corn plant. For example, the heterologous DNA may itself comprise or consist of a promoter that is active in *Zea mays*, or may utilize a promoter already present in the *Zea mays* genotype that is the transformation target.

The compositions of and method for constructing heterologous DNA which can transform certain plants is well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the heterologous DNA useful herein. The specific composition

of the DNA is not central to the present invention and the invention is not dependent upon the composition of the specific transforming DNA used. Weising et al. (1988), the subject matter of which is incorporated herein by reference, describes suitable DNA components, which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, as well as provides suitable references for compositions therefrom e.g., on Table 1.

Suitable heterologous DNA for use herein includes all DNA which provides for, or enhances, a beneficial feature of the resultant transgenic corn plant. The DNA may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. For example, the DNA can encode a bacterial dad A for increased lysine production; *Bacillus thuringiensis* (BT) t-endotoxin or protease inhibitor for insect resistance; bacterial EPSP synthase for resistance to glyphosate herbicide; and chitinase or glucan endo-1,3-B-glucosidase for fungicidal properties. Aside from DNA sequences that serve as transcription units or portions thereof, useful DNA may be untranscribed, serving a regulatory or a structural function. Also, the DNA may be introduced to act as a genetic tool to generate mutants and/or

TABLE 1

Selectable marker and reporter genes in plant genetic transformation

Gene	Origin	Encoded enzyme	Useful as: Selectable marker	Useful as: Scorable reporter	Resistance against
Neomycin phosphotransferase gene II (nptII)	Tn5	neomycin phosphotransferase	++	+	neomycin kanamycin G-418[1]
Neomycin phosphotransferase gene I (nptI)	Nn601	neomycin phosphotransferase	+	+	neomycin kanamycin G-418[2]
Chloramphenicol acetyltransferase gene (cat)	Tn9	chloramphenicol acetyltransferase	(+)	++	chloramphenicol[3]
Bacterial DHFR gene	plasmid R67	dihydrofolate reductase	+	+	methotrexate[4]
Mutated c-DNA of a mouse DHFR gene	mouse	dihydrofolate reductase	++	+	methotrexate[5]
Octopine synthase gene (ocs)	T-DNA	octopine synthase	+	++	toxic opine precursor analogues, i.e. aminoethylcystein[6]
Nopaline synthase gene (nos)	T-DNA	nopaline synthase	–	++	—[7]
Hygromycin phosphotransferase gene (hpt)	*E. coli*	hygromycin phosphotransferase	++	–	hygromycin B[8]
Bleomycin resistance gene	Tn5	?	+	–	bleomycin[9]
Streptomycin phosphotransferase gene	Tn5	streptomycin phosphotransferase	(+)	(+)	streptomycin[10]
			control plants are not killed by streptomycin		
aroA gene	*Salmonella typhimurium*	EPSP synthase	++	–	glyphosate[11]
bar gene	*Streptomices hygroscopicus*	phosphinothricin acetyltransferase	++	–	phosphinothricin, bialophos[12]
β-galactosidase gene	*E. coli*	β-galactosidase	–	+	—[13]
Glucuronidase gene (GUS)	*E. coli*	glucuronidase	–	++	—[14]
Bacterial luciterase gene	*Vibrso harvest*	luciferase	–	++	—[15]
Firefly luciferase gene	*Photonus peralis*	luciferase	–	++	—[16]

Only some representative references were chosen in case of nptII, nos, ocs and cat genes.
Abbreviations
Tn - transpuson
DHFR - dihydrofolate reductase
EPSP synthase - 5-enolpyruvylskikimate-3-phosphate synthase
[1]M. Bevrin et al., Nature, 304, 185 (1983); M. DeBlock et al., EMBO J., 8, 1681 (1984); I. Herrera-Estrella et al., EMBO J., 2, 987 (1983).
[2]R. T. Fraley et al., PNAS USA, 80, 1803 (1983); H. Pretzak et al., Nucl. Acids Res., 14, 5857 (1986).
[3]M. DeBlock et al., EMBO J., 3, 1681 (1984); I. Herrera-Estrella et al., Nature, 303, 209 (1983).
[4]N. Brisson et al., Nature, 310, 511 (1984); M. DeBlock et al., ibid., I. Herrera-Estrella et al., EMBO J., 2, 987 (1983)
[5]D. A. Eichholtz et al., Somat. Cell. Mol. Genct., 13, 67 (1987).
[6]G. A. Dahl et al., Theor. Appl. Genct., 66, 233 (1983); H. De Geve et al., Nature, 300, 752 (1982); A. Hockema et al., Plant Mol. Biol., 5, 85 (1985); M. G. Koztel et al., J. Mol. Appl. Genct., 2, 549 (1981).
[7]J. D. G. Jones et al., EMBO J., 4, 2411 (1985); C. H. Shaw et al., Nuc. Acids Res., 14, 6003 (1986); P. Zambryska et al., EMBO J., 2, 2443 (1983).
[8]A. M. Lloyd et al., Science, 284, 464 (1986); P. I. M. Van den Hazen et al., Plant MOL Biol., 5, 299 (1985); C. Waldron et al., Plant Mol. Biol., 5, 103 (1985).
[9]J. Hille et al., Plant Mol. Biol., 7, 171 (1986).
[10]J. D. G. Jones et al., Mol. Gen. Genet., 210, 86 (1987).
[11]L. Comai et al., Nature, 317, 741 (1985); J. J. Inllatti et al., Biotechnology, 5, 726 (1987).
[12]M. DeBlock et al., EMBO J., 6, 2513 (1987); C. I. Thompson et al., EMBO J., 6, 2519 (1987).
[13]G. Heimer et al., Biotechnology, 2, 520 (1984).
[14]D. R. Gallie et al., Nuc. Acids Res., 15, 8693 (1987); R. A. Jefferson et al., EMBO J., 6, 1901 (1987).
[15]C. Koncz et al., Mol. Gen. Genet., 204, 383 (1986).
[16]D. W. Ow et al., Science, 234, 856 (1986); D. W. Ow et al., PNAS USA, 84, 4870 (1987); C. D. Riggs et al., Nucl. Acids Res., 15, 8115 (1987).

Sambrook et al. (1989) provides suitable methods of construction.

Generally, the heterologous DNA will be relatively small, i.e., less than about 30 Kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the DNA increases.

assist in the identification, genetic tagging, or isolation of segments of corn DNA. Additional examples may be found in Weising, supra.

The heterologous DNA to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a co-transformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Weising et al., supra. A preferred selectable marker gene is the hygromycin B phosphotransferase (HPT) coding sequence, which may be derived from *E. coli* and which confers resistance to the antibiotic hygromycin B. Other selectable markers include aminoglycoside phosphortansferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin, and G418, as well as those genes which code for resistance or tolerance to glyphosate, 1,2-dichloropropionic acid methotrexate, imidazolinones, sulfonylureas, bromoxynil, phosphonothricin and the like. Those selectable marker genes which confer herbicide resistance or tolerance are also of commercial utility in the resulting transformed plants.

Reporter genes which encode for easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present in or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g., phenotypic change or enzymatic activity. Examples of such genes are provided in Weising et al., supra. Preferred genes include the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the beta-glucuronidase gene of the uidA locus of *E. coli*, and the luciferase genes from firefly *Photinus pyralis*.

The regulatory sequences useful herein include any constitutive, inducible, tissue or organ specific, or developmental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al., supra. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *Agrobacterium tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase promoter from corn; light inducible promoters such as, ribulose-biphosphate-carboxylase small subunit gene from a variety of species; and the major chlorophyll a/b binding protein gene promoter; 35S and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ-specific expression or expression at specific development stage(s) of the plant.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present on the DNA. Such elements may or may not be necessary for the function of the DNA, although they can provide a better expression or functioning of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the plant. For example, the maize Adh1S first intron may be placed between the promoter and the coding sequence of a particular heterologous DNA. This intron, when included in a DNA construction, is known to generally increase expression of a protein in maize cells. (Callis et al. 1987) However, sufficient expression for a selectable marker to perform satisfactorily can often be obtained without an intron. (Klein et al. 1989) An example of an alternative suitable intron is the *shrunken*-1 first intron of *Zea mays*. These other elements must be compatible with the remainder of the DNA constructions.

To determine whether a particular combination of DNA and recipient plant cells are suitable for use herein, the DNA may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the DNA has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (GUS) gene (Jefferson et al. 1987). In the case of the microprojectile bombardment transformation process of the present invention, a suitable time for conducting the assay is about 2–3 days after bombardment. The use of transient assays is particularly important when using DNA components which have not previously been demonstrated or confirmed as compatible with the desired recipient cells.

III. DNA Delivery Process

The DNA can be introduced into the regenerable maize cell cultures, preferably into callus cultures via a particle bombardment process. A general description of a suitable particle bombardment instrument is provided in Sanford et al. (1987), the disclosure of which is incorporated herein by reference. While protocols for the use of the instrument in the bombardment of maize non-regenerable suspension culture cells are described in Klein et al. (1988a, 1988b, and 1989), no protocols have been published for the bombardment of callus cultures or regenerable maize cells.

In a microprojectile bombardment process, also referred to as a biolistic process, the transport of the DNA into the callus is mediated by very small particles of a biologically inert material. When the inert particles are coated with DNA and accelerated to a suitable velocity, one or more of the particles is able to enter into one or more of the cells where the DNA is released from the particle and expressed within the cell. While some of the cells are fatally damaged by the bombardment process, some of the recipient cells do survive, stably retain the introduced DNA, and express it.

The particles, called microprojectiles, are generally of a high density material such as tungsten or gold. They are coated with the DNA of interest. The microprojectiles are then placed onto the surface of a macroprojectile which serves to transfer the motive force from a suitable energy source to the microprojectiles. After the macroprojectile and the microprojectiles are accelerated to the proper velocity, they contact a blocking device which prevents the macroprojectile from continuing its forward path but allows the DNA-coated microprojectiles to continue on and impact the recipient callus cells. Suitable such instruments may use a variety of motive forces such as gunpowder or shock waves from an electric arc discharge (Swain et al. 1988). An instrument in which gunpowder is the motive force is currently preferred and such is described and further explained in Sanford et al. (1987), the disclosure of which is incorporated herein by reference.

A protocol for the use of the gunpowder instrument is provided in Klein et al. (1988a, b) and involves two major steps. First, tungsten microprojectiles are mixed with the DNA, calcium chloride, and spermidine free base in a

specified order in an aqueous solution. The concentrations of the various components may be varied as taught. The preferred procedure entails exactly the procedure of Klein et al. (1988b) except for doubling the stated optimum DNA concentration. Secondly, the DNA-coated microprojectiles, macroprojectiles, and recipient cells are placed in position in the instrument and the motive force is applied to the macroprojectiles. Parts of this step which may be varied include the distance of the recipient cells from the end of the barrel as well as the vacuum in the sample chamber. The recipient tissue is positioned 5 cm below the stopping plate tray.

The callus cultures useful herein for generation of transgenic plants should generally be about midway between transfer periods, and thus, past any "lag" phase that might be associated with a transfer to a new media, but also before reaching any "stationary" phase associated with a long time on the same plate. The specific tissue subjected to the bombardment process is preferably taken about 7–10 days after subculture, though this is not believed critical. The tissue should generally be used in the form of pieces of about 30 to 80, preferably about 40 to 60, mg. The clumps are placed on a petri dish or other surface and arranged in essentially any manner, recognizing that (i) the space in the center of the dish will receive the heaviest concentration of metal-DNA particles and the tissue located there is likely to suffer damage during bombardment and, (ii) the number of particles reaching a cell will decrease (probably exponentially) with increasing distance of the cell from the center of the blast so that cells far from the center of the dish are not likely to be bombarded and transformed. A mesh screen, preferably of metal, may be laid on the dish to prevent splashing or ejection of the tissue. The tissue may be bombarded one or more times with the DNA-coated metal particles.

IV. Selection Process

Once the calli have been bombarded with the DNA and the DNA has penetrated some of the cells, it is necessary to identify and select those cells which both contain the heterologous DNA and still retain sufficient regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively, and preferably, when a selectable marker gene has been transmitted along with or as part of the heterologous DNA, those cells of the callus which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells while simultaneously inhibiting the growth of the non-transformed cells. The situation is complicated by the fact that the vitality of individual cells in a population is often highly dependent on the vitality of neighboring cells. Also, the selection conditions must not be so severe that the plant regeneration capacity of the callus cells and the fertility of the resulting plant are precluded. Thus, the effects of the selection agent on cell viability and morphology should be evaluated. This may be accomplished by experimentally producing a growth inhibition curve for the given selective agent and tissue being transformed beforehand. This will establish the concentration range which will inhibit growth.

When a selectable marker gene has been used, the callus clumps may be either allowed to recover from the bombardment on non-selective media, or preferably, directly transferred to media containing that agent.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably, the concentration of the agent is initially such that about a 5–40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells to preferentially grow and divide while inhibiting untransformed cells, but not to the extent that growth of the transformed cells is prevented. Once the few individual transformed cells have grown sufficiently, the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of non-transformed cells habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative maize transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells. The callus may also be cultured on non-selective media at various times during the overall selection procedure.

Once a callus sector is identified as a putative transformant, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e., Southern blot or polymerase chain reaction (PCR) or the like.

V. Regeneration of Plants and Production of Seed

Cell lines which have been shown to be transformed must then be regenerated into plants and the fertility of the resultant plants determined. Transformed lines which test positive by genotypic and/or phenotypic analysis are then placed on a media which promotes tissue differentiation and plant regeneration. Regeneration may be carried out in accordance with standard procedures well known in the art. The procedures commonly entail reducing the level of auxin which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. One example of such a regeneration procedure is described

in Green et al. (1982). The plants are grown to maturity in a growth room or greenhouse and appropriate sexual crosses and selfs are made as described by Neuffer (1982).

Regeneration, while important to the present invention, may be performed in any conventional manner. If a selectable marker has been transformed into the cells, the selection agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are transformed. Since regeneration techniques are well known and not critical to the present invention, any technique which accomplishes the regeneration and produces fertile plants may be used.

VI. Analysis of R1 Progeny

The plants regenerated from the transformed callus are referred to as the RO generation or RO plants. The seeds produced by various sexual crosses of the RO generation plants are referred to as R1 progeny or the R1 generation. When R1 seeds are germinated, the resulting plants are also referred to as the R1 generation.

To confirm the successful transmission and inheritance of the heterologous DNA in the sexual crosses described above, the R1 generation should be analyzed to confirm the presence of the transforming DNA. The analysis may be performed in any of the manners such as were disclosed above for analyzing the bombarded callus for evidence of transformation, taking into account the fact that plants and plant parts are being used in place of the callus.

VII. Establishment of the Heterologous DNA in Other Maize Varieties

Fertile, transgenic plants may then be used in a conventional maize breeding program in order to incorporate the introduced heterologous DNA into the desired lines or varieties. Conventional breeding programs employ a conversion process (backcrossing). Methods and references for convergent improvement of corn are given by Hallauer et al., (1988) incorporated herein by reference. Briefly, conversion is performed by crossing the initial transgenic fertile plant to normal elite inbred lines. The progeny from this cross will segregate such that some of the plants will carry the heterologous DNA whereas some will not. The plants that do carry the DNA are then crossed again to the normal plant resulting in progeny which segregate once more. This backcrossing process is repeated until the original normal parent has been converted to a line containing the heterologous DNA and also possessing all other important attributes originally found in the parent. Generally, this will require about 6–8 generations. A separate backcrossing program will be generally used for every elite line that is to be converted to a genetically engineered elite line.

Generally, the commercial value of the transformed corn produced herein will be greatest if the heterologous DNA can be incorporated into many different hybrid combinations. A farmer typically grows several varieties of hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based upon his physical location since hybrids adapted to one part of the corn belt are generally not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the heterologous DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable heterologous DNA.

Corn breeding and the techniques and skills required to transfer genes from one line or variety to another are well known to those skilled in the art. Thus, introducing heterologous DNA into other lines or varieties can be readily accomplished by these breeding procedures whether or not they generate the appropriate calli.

VIII. Uses of Transgenic Plants

The transgenic plants produced herein are expected to be useful for a variety of commercial and research purposes. Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g., agronomic traits such as pest resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g., improved nutritive content in human food or animal feed), or beneficial to the food processor (e.g., improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods. However, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silage or for ornamental purposes (e.g., Indian corn). Often, chemical constituents (e.g., oils or starches) of corn and other crops are extracted for foods or industrial use and transgenic plants may be created which have enhanced or modified levels of such components. The plants may also be used for seed production for a variety of purposes.

Transgenic plants may also find use in the commercial manufacture of proteins or other molecules encoded by the heterologous DNA contained therein, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules, or for other purposes (e.g., for research).

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded by the heterologous DNA may be transferred, e.g., from corn cells to cell of other species, e.g., by protoplast fusion.

The transgenic plants may have many uses in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. The methods of the invention may also be used to create plants having unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties.

The following non-limiting examples are illustrative of the present invention. They are presented to better explain the general procedures which were used to prepare the fertile *Zea mays* plants of this invention which stably express the heterologous DNA and which transmit that DNA to progeny. All parts and percents are by weight unless otherwise specified. It must be recognized that a specific transformation event is a function of the amount of material subjected to the transformation procedure. Thus, when individual situations arise in which the procedures described herein do not produce a transformed product, repetition of the procedures will be required.

EXAMPLE I.

Fertile transgenic *Zea mays* plants which contain heterologous DNA which is heritable were prepared as follows:

I. Initiation and maintenance of maize cell cultures which retain plant regeneration capacity

Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred line A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile, distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient medium initiation/maintenance media with the root/shoot axis exposed to the medium. Initiation/maintenance media (hereinafter referred to as "F medium") consisted of N6 basal media (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc., San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

The immature embryos were incubated at 26° C. in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well-defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh media. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell culture named AB12 used in this example was such a culture and had been initiated about 1 year before bombardment.

II. Plasmids—pCHN1-1, pHYGI1, pBII221, and pLUC-1

The plasmids pCHN1-1, pHYGI1, and pLUC-1 were constructed in the vector pBS+ (Stratagene, Inc., San Diego, Calif.), a 3.2 Kb circular plasmid, using standard recombinant DNA techniques. pCHN1-1 contains the hygromycin B phosphotransferase (HPT) coding sequence from *E. coli* (Gritz et al. 1983) flanked at the 3' end by the nopaline synthase (nos) polyadenylation sequence of *Agrobacterium tumefaciens* (Chilton and Barnes 1983). Expression is driven by the cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982), located upstream from the hygromycin coding sequence. The plasmid pHYGI1 was constructed by inserting the 553 bp Bcl-BamHI fragment containing the maize AdhIS first intron (Callis et al. 1987) between the CaMV 35S promoter and the hygromycin coding sequence of pCHN1-1. A map of pHYGI1 is provided as FIG. 1. A sample of pHYGI1 was deposited at the American Type Culture Collection, Rockville, Md., U.S.A., on Mar. 16, 1990, under the provisions of the Budapest Treaty, and assigned accession number 40774.

pBII221 contains the *E. coli* B-glucuronidase coding sequence flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. The plasmid was constructed by inserting the maize AdhIS first intron between the 35S promoter and the coding sequence of pBI221 (Jefferson et al. 1987). A map of pBII221 is provided as FIG. 2.

pLUC-1 contains the firefly luciferase coding sequence (DeWet et al. 1987) flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. This plasmid was used solely as negative control DNA.

Plasmids were introduced into the embryogenic callus culture AB12 by microprojectile bombardment.

III. DNA delivery process

The embryogenic maize callus line AB12 was subcultured 7 to 12 days prior to microprojectile bombardment. AB12 was prepared for bombardment as follows. Five clumps of callus, each approximately 50 mg in wet weight were arranged in a cross pattern in the center of a sterile 60×15 mm petri plate (Falcon 1007). Plates were stored in a closed container with moist paper towels, throughout the bombardment process. Twenty-six plates were prepared.

Plasmids were coated onto M-10 tungsten particles (Biolistics) exactly as described by Klein et al. (1988b) except that, (i) twice the recommended quantity of DNA was used, (ii) the DNA precipitation onto the particles was performed at 0° C., and (iii) the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process.

All of the tubes contained 25 µl 50 mg/ml M-10 tungsten in water, 25 µl 2.5M $CaCl_2$, and 10 µl 100 mM spermidine free base along with a total of 5 µl 1 mg/ml total plasmid content. When two plasmids were used simultaneously, each was present in an amount of 2.5 µl. One tube contained only plasmid pBII221; two tubes contained both plasmids pHYGI1 and pBII221; two tubes contained both plasmids pCHN1-1 and pBII221; and one rude contained only plasmid pLUC-1.

All tubes were incubated on ice for 10 min., pelletized by centrifugation in an Eppendorf centrifuge at room temperature for 5 seconds, and 25 µl of the supernatant was discarded. The tubes were stored on ice throughout the bombardment process. Each preparation was used for no more than 5 bombardments.

Macroprojectiles and stopping plates were obtained from Biolistics, Inc. (Ithaca, N.Y.). They were sterilized as described by the supplier. The microprojectile bombardment instrument was obtained from Biolistics, Inc.

The sample plate tray was positioned at the position 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate in the slot nearest to the barrel. Plates of callus tissue prepared as described above were centered on the sample plate tray and the petri dish lid removed. A 7×7 cm square rigid wire mesh with 3×3 mm mesh and made of galvanized steel was placed over the open dish in order to retain the tissue during the bombardment. Tungsten/DNA preparation were sonicated as described by Biolistics, Inc. and 2.5 µl was pipetted onto the top of the macroprojectiles. The instrument was operated as described by the manufacturer. The bombardments which were performed are summarized on Table 2.

TABLE 2

2 × pBII221 prep	To determine transient expression frequency
10 × pHYGI1/pBII221	As a potential positive treatment for transformation
10 × pCHN1-1/pBII221	As a potential positive treatment for transformation
4 × pLUC-1	Negative control treatment

The two plates of callus bombarded with pBII221 were transferred plate for plate to F medium (with no hygromycin) and the callus cultured at 26° C. in the dark. After 2 days, this callus was then transferred plate for plate into

35×10 mm petri plates (Falcon 1008) containing 2 ml of GUS assay buffer which consists of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (Research Organics), 100 mM sodium phosphate pH 7.0, 5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM EDTA, and 0.06% Triton X-100. These were incubated at 37° C. for 3 days later which the number of blue cells was counted giving 291 and 477 transient GUS expressing cells in the two plates, suggesting that the DNA delivery process had also occurred with the other bombarded plates. These plates were discarded after counting since the GUS assay is destructive.

IV. Selection process

Hygromycin a (Calbiochem) was incorporated into the medium by addition of the appropriate volume of filter sterilized 100 mg/ml hygromycin B in water when the media had cooled to 45° C. prior to pouring plates.

Immediately after all samples had been bombarded, callus from all of she plates treated with pHYGI1/pBII221, pCHN1-1/pBII221 and three of the plates treated with pLUC-1 were transferred plate for plate onto F medium containing 15 mg/l hygromycin B, (five pieces of callus per plate). These are referred no as round 1 selection plates. Callus from the fourth plate treated with pLUC-1 was transferred to F medium without hygromycin. This tissue was subcultured every 2–3 weeks onto nonselective medium and is referred to as unselected control callus.

After two weeks of selection, tissue appeared essentially identical on both selective and nonselective media. All callus from eight plates from each of the pHYGI1/pBII221 and pCHN1-1/pBII221 treatments and two plates of the control callus on selective media were transferred from round 1 selection plates to round 2 selection plates that contained 60 mg/l hygromycin. The round 2 selection plates each contained ten 30 mg pieces of callus per plate, resulting in an expansion of the total number of plates.

The remaining tissue on selective media, two plates each of pHYGI1/pBII221 and pCHN1-1/pBII221 treated tissue and one of control callus, were placed in GUS assay buffer at 37° C. to determine whether blue clusters of cells were observable at two weeks post-bombardment. After 6 days in assay buffer, this tissue was scored for GUS expression. The results are summarized on Table 3.

TABLE 3

Treatment	Replicate	Observations
pLUC-1		No blue cells
pHYGI1/pBII221	Plate 1	11 single cells 1 four-cell cluster
	Plate 2	5 single cells
pCHN1-1/pBII221	Plate 1	1 single cell 2 two-cell clusters
	Plate 2	5 single cells 1 two-cell cluster 2 clusters of 8–10 cells

After 21 days on the round 2 selection plates, all viable portions of the material were transferred to round 3 selection plates containing 60 mg/l hygromycin. The round 2 selection plates, containing only tissue that was apparently dead, were reserved. Both round 2 and 3 selection plates were observed periodically for viable proliferating sectors.

After 35 days on round 3 selection plates, both the round 2 and round 3 sets of selection plates were checked for viable sectors of callus. Two such sectors were observed proliferating from a background of dead tissue on plates treated with pHYGI1/pBII221. The first sector named 3AA was from the round 3 group of plates and the second sector named 6L was from the round 2 group of plates. Both lines were then transferred to F medium without hygromycin.

After 19 days on F medium without hygromycin, the line 3AA grew very little whereas the line 6L grew rapidly. Both were transferred again to F medium for 9 days. The lines 3AA and 6L were then transferred to F medium containing 15 mg/l hygromycin for 14 days. At this point, line 3AA was observed to be of very poor quality and slow growing. The line 6L, however, grew rapidly on F medium with 15 mg/l hygromycin; the line was then subcultured to F medium without hygromycin.

After 10 days on F medium, an inhibition study of the line 6L was initiated. Callus of 6L was transferred onto F medium containing 1, 10, 30, 100, and 250 mg/l hygromycin B. Five plates of callus were prepared for each concentration and each plate contained ten approximately 50 mg pieces of callus. One plate of unselected control tissue was prepared for each concentration of hygromycin.

It was found that the line 6L was capable of sustained growth over 9 subcultures on 0, 10, 30, 100, and 250 mg/l hygromycin. The name of the line 6L was changed at this time from 6L to PH1 (Positive Hygromycin transformant 1).

Additional sectors were recovered at various time points from the round 2 and 3 selection plates. None of these were able to grow in the presence of hygromycin for multiple rounds, i.e., two or three subcultures.

V. Confirmation of transformed callus

To show that the PH1 callus had acquired the hygromycin resistance gene, a Southern blot of PH1 callus was prepared as follows: DNA was isolated from PH1 and unselected control calli by freezing 2 g of callus in liquid nitrogen and grinding it to a fine powder which was transferred to a 30 ml Oak Ridge tube containing 6 ml extraction buffer (7M urea, 250 mM NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1% sarcosine). To this was added 7 ml of phenol:chloroform 1:1, the tubes shaken and incubated at 37° C. 15 min. Samples were centrifuged at 8K for 10 min. at 4° C. The supernatant was pipetted through miracloth (Calbiochem 475855) into a disposable 15 ml tube (American Scientific Products, C3920-15A) containing 1 ml 4.4M ammonium acetate, pH 5.2. Isopropanol, 6 ml was added, the tubes shaken, and the samples incubated at –20° C. for 15 min. The DNA was pelleted in a Backman TJ-6 centrifuge at the maximum speed for 5 min. at 4° C. The supernatant was discarded and the pellet was dissolved in 500 µl TE-10 (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) 15 min. at room temperature. The samples were transferred to a 1.5 ml Eppendorf tube and 100 µl 4.4M ammonium acetate, pH 5.2 and 700 µl isopropanol were added. This was incubated at –20° C. for 15 min. and the DNA pelleted 5 min. in an Eppendorf microcentrifuge (12,000 rpm). The pellet was washed with 70% ethanol, dried, and resuspended in TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The isolated DNA (10 µg) was digested with BamHI (NEB) and electrophoresed in a 0.8% w/v agarose gel at 15 V for 16 hrs in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The DNA within the gel was then depurinated by soaking the gel twice in 0.25 HCl for 15 min., denatured and cleaved by soaking the get twice in 0.5M NaOH/1.0M NaCl 15 min., and neutralized by soaking the gel twice in 0.5M

Tris pH 7.4/3 M NaCl 30 min. DNA was then blotted onto a Nytran membrane (Shleicher & Shuell) by capillary transfer overnight in 6× SSC (20× SSC, 3M NaCl, 0.3M sodium citrate pH 7.0). The membrane was baked at 80° C. for 2 hrs under vacuum. Prehybridization treatment of the membrane was done in 6× SSC, 10× Denhardt's solution, 1% SDS, 50 μg/ml denatured salmon sperm DNA using 0.25 ml prehybridization solution per cm^2 of membrane. Prehybridization was carried out at 42° C. overnight.

A ^{32}P labelled probe was prepared by random primer labelling with an Oligo Labelling Kit (Pharmacia) as per the supplier's instructions with ^{32}P-dCTP (ICN Radiochemicals). The template DNA used was the 1055 bp BamHI fragment of pHYGI1, which is the HPT coding sequence. The fragment was gel purified and cut again with PstI (NEB) before labelling.

The hybridization was performed in 50% formamide, 6× SSC, 1% SDS, 50 μ/ml denatured salmon sperm DNA (Sigma), 0.05% sodium pyrophosphate and all of the isopropanol precipitated heat denatured probe (10^7 CPM/50 ng template). The hybridization was carried out at 42° C. overnight.

The membrane was washed twice in 50 ml 6× SSC, 0.1% SDS 5 min. at room temperature with shaking, then twice in 500 ml 6× SSC, 0.1% SDS 15 min at room temperature, then twice in 500 ml 1× SSC, 1% SDS 30 min at 42° C., and finally in 500 ml 0.1× SSC, 1% SDS 60 min. at 65° C. Membranes were exposed to Kodak X-OMAT AR film in an X-OMATIC cassette with intensifying screens. As shown in FIG. **3**, a band was observed for PH1 callus at the expected position of 1.05 Kb, indicating that the HPT coding sequence was present. No band was observed for control callus.

To demonstrate that the hygromycin gene is incorporated into high molecular weight DNA, DNA isolated from PH1 callus and control callus was treated with (i) no restriction enzyme, (ii) BamHI, as described previously, or (iii) PstI, which cuts the plasmid pHYGI1 only once within the HPT coding sequence. Samples were blotted and probed with the HPT coding sequence as described previously.

Undigested PH1 DNA only showed hybridization to the probe at the position of uncut DNA, demonstrating that the hygromycin gene is incorporated into high molecular weight DNA. The expected 1.05 Kb band for PH1 DNA digested with BamHi was observed, as had been shown previously. For PH1 DNA digested with PstI, a 5.9 Kb band would be expected if the hygromycin gene was present on an intact pHYGI1 plasmid. Two or more bands of variable size (size dependent on the position flanking PstI sites within the host DNA would be expected if the gene was incorporated into high molecular weight DNA. Three bands were observed with approximate molecular sizes of 12, 5.1, and 4.9 Kb. This result demonstrates incorporation of the hygromycin gene into high molecular weight DNA. The intensity of the 4.9 Kb band is approximately twice as great as the other two bands, suggesting either partial digestion or possibly a tandem repeat of the HPT gene. No hybridization was observed for DNA from control callus in any of the above treatments.

These results prove that the HPT coding sequence is not present in PH1 callus as intact pHYGI1 or as a small non-chromosomal plasmid. They are consistent with incorporation of the hygromycin gone into high molecular weight DNA.

VI. Plant regeneration and production of seed

PH1 callus was transferred directly from all of the concentrations of hygromycin used in the inhibition study to RM5 medium which consists of MS basal salts (Murashige et al. 1962) supplemented with thiamine.HCl 0.5 mg/l, 2,4-D 0.75 mg/l sucrose 50 g/l, asparagine 150 mg/l, and Gelrite 2.5 g/l (Kelco Inc., San Diego).

After 14 days on RM5 medium, the majority of PH1 and negative control callus was transferred to R5 medium which is the same as RM5 medium, except that 2,4-D omitted. These were cultured in the dark for 7 days at 26° C. and transferred to a light regime of 14 hrs light and 10 hrs dark for 14 days at 26° C. At this point, plantlets that had formed were transferred to one quart canning jars (Ball) containing 100 ml of R5 medium. Plants were transferred from jars to vermiculite for 7 or 8 days before transplanting then into soil and growing them to maturity. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the Ro tissue, a Southern blot was performed as previously described on BamHI digested leaf DNA from three randomly chosen Ro plants of PH1. As shown in FIG. **4**, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred *Zea mays* lines A188, B73, and Oh43. Seed was harvested 45 days post-pollination and allowed to dry further 1–2 weeks. Seed set varied from 0 to 40 seeds per ear when PH1 was the female parent and 0 to 32 seeds per ear when PH1 was the male parent.

VII. Analysis of the R1 progeny

The presence of the hygromycin resistance trait was evaluated by a root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears.

A. Root elongation bioassay

Seed was sterilized in a 1:1 dilution of commercial bleach in water plus alconox 0.1% for 20 min. in 125 ml Erlenmyer flasks and rinsed 3 times in sterile water and inbibed overnight in sterile water containing 50 mg/ml captan by shaking at 150 rpm.

After imbibition, the solution was decanted from the flasks and the seed transferred to flow boxes (Flow Laboratories) containing 3 sheets of H_2O saturated germination paper. A fourth sheet of water saturated germination paper was placed on top of the seed. Seed was allowed to germinate 4 days.

After the seed had germinated, approximately 1 cm of the primary root tip was excised from each seedling and plated on MS salts, 20 g/l sucrose, 50 mg/l hygromycin, 0.25% Gelrite, and incubated in the dark at 26° C. for 4 days.

Roots were evaluated for the presence or absence of abundant root hairs and root branches. Roots were classified as transgenic (hygromycin resistant) if they had root hairs and root branches, and untransformed (hygromycin sensitive) if they had limited numbers of branches. The results are shown in Table 3, hereinbelow.

B. Etiolated leaf bioassay

After the root tips were excised as described above, the seedlings of one PH1 ear and one control ear were transferred to moist vermiculite and grown in the dark for 5 days.

At this point, 1 mm sections were cut from the tip of the coleoptile, surface sterilized 10 seconds, and plated on MS basal salts, 20 g/l sucrose, 2.5 g/l Gelrite with either 0 (control) or 100 mg/l hygromycin and incubated in the dark at 26° C. for 18 hrs. Each plate contained duplicate sections of each shoot. They were then incubated in a light regimen of 14 hrs light 10 hrs dark at 26° C. for 48 hrs, and rated on a scale of from 0 (all brown) to 6 (all green) for the percent of green color in the leaf tissue. Shoots were classified as untransformed (hygromycin sensitive) if they had a rating of zero and classified as transformed (hygromycin resistant) if they had a rating of 3 or greater. The results are shown in Table 1, hereinbelow.

C. Southern blots

Seedlings from the bioassays were transplanted to soil and were grown to sexual maturity. DNA was isolated from 0.8 g of leaf tissue about 3 weeks after transplanting to soil and probed with the HPT coding sequence as described previously. Plants with a 1.05 Kb band present in the Southern blot were classified as transgenic. As shown in FIG. **5**, two out of seven progeny of PH1 plant 3 were transgenic as were three out of eight progeny of PH1 plant 10. The blot results correlated precisely with data from the bioassays, confirming that the heterologous DNA was transmitted through one complete sexual life cycle. All data are summarized in Table 4.

TABLE 4

	ANALYSIS OF PH1 R1 PLANTS						
PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.1	+	ND	+	4.1	–	ND	ND
3.2	–	ND	–	4.2	–	ND	ND
3.3	–	ND	–	4.3	–	ND	ND
3.4	–	ND	–	4.4	–	ND	ND
3.5	–	ND	–	4.5	–	ND	ND
3.6	+	ND	+	4.6	–	ND	ND
3.7	–	ND	–	4.7	–	ND	ND
				2.1	–	ND	–
10.1	+	+	+	1.1	–	–	–
10.2	+	+	+	1.2	–	–	ND
10.3	–	–	ND	1.3	–	–	ND
10.4	–	–	–	1.4	–	–	ND
10.5	–	–	–	1.5	–	–	ND
10.6	–	–	–	1.6	–	–	ND
10.7	–	–	–	1.7	–	–	ND
10.8	ND	+	+	1.8	–	–	ND

Key: + transgenic; – = nontransgenic; ND = not done

EXAMPLE II

The procedure of Example I was repeated with minor modifications.

I. Plant Lines and Tissue Cultures

The embryogenic maize callus line, AB12, was used as in Example I. The line had been initiated about 18 months before the actual bombardment occurred.

II. Plasmids

The plasmids pBII221 and pHYGI1 described in Example I were used.

III. DNA Delivery Process

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. All of the tubes contained 25 µl 50 mg/ml M-10 tungsten in water, 25 µl 2.5M $CaCl_2$, and 10 µl 100 mM spermidine free base along with a total of 5 µl 1 mg/ml total plasmid content. One tube contained only plasmid pBII221; two tubes contained only plasmid pHYGI1; and one tube contained no plasmid but 5 µl TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done: 2×pBII221 prep (for transient expression); 7×pHYGI1 prep (potential positive treatment); and 3×TE prep (negative control treatment).

After all the bombardments were performed, the callus from the pBII221 treatment was transferred plate for plate to F medium as five 50 mg pieces. After 2 days, the callus was placed into GUS assay buffer as per Example I. Numbers of transiently expressing cells were counted and found to be 686 and 845 GUS positive cells, suggesting that the particle delivery process had occurred in the other bombarded plates.

IV. Selection of Transformed Callus

After bombardment, the callus from the pHYGI1 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate (different from Example I). The same was done for two of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue (unselected control callus).

After 13 days, the callus on round 1 selection plates was indistinguishable from unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin. An approximate five-fold expansion of the numbers of plates occurred.

The callus on round 2 selection plates had increased substantially in weight after 23 days, but at this time appeared close to dead. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin. This transfer of all material from round 2 to round 3 selection differs from Example I in which only viable sectors were transferred and the round 2 plates reserved.

At 58 days post-bombardment, three live sectors were observed proliferating from the surrounding dead tissue. All three lines were from pHYGI1 treatments and were designated 24C, 56A, and 55A.

After 15 days on maintenance medium, growth of the lines was observed. The line 24C grew well whereas lines 55A and 56A grew more slowly. All three lines were transferred to F medium containing 60 mg/l hygromycin. Unselected control callus from maintenance medium was plated to F medium having 60 mg/l hygromycin.

After 19 days on 60 mg/l hygromycin, the growth of line 24C appeared to be entirely uninhibited, with the control showing approximately 80% of the weight gain of 24C. The line 56A was completely dead, and the line 55A was very close to dead. The lines 24C and 55A were transferred again to F 60 mg/l hygromycin as was the control tissue.

After 23 days on 60 mg/l hygromycin, the line 24C again appeared entirely uninhibited. The line 55A was completely dead, as was the negative control callus on its second exposure to F medium having 60 mg/l hygromycin.

At 88 days post-bombardment, a sector was observed proliferating among the surrounding dead tissue on the round 3 selection plates. The callus was from a plate bombarded with pHYGI1 and was designated 13E. The callus was transferred to F medium and cultured for 19 days. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin, and (ii) F medium containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin. After 14 days of culture, the callus line 13E appeared uninhibited on both levels of hygromycin. The control callus appeared to have about 80% of the weight gain of 13E. The callus lines were transferred to fresh media at the same respective levels of hygromycin.

V. Confirmation of Transformed Callus

A Southern blot was prepared from BamHI-digested DNA from the line 24C. As shown in FIG. 6, a band was observed for the line 24C at the expected size of 1.05 Kb showing that the line 24C contained the HPT coding sequence. No band was observed for DNA from control tissue. The name of the callus line 24C was changed to PH2.

To demonstrate that the hygromycin gene is incorporated into high molecular weight DNA, DNA isolated from PH2 callus and control callus was treated with (i) no restriction enzyme, (ii) BamHI, as described previously, or, (iii) PstI, which cuts the plasmid pHYGI1 only once within the HPT coding sequence. Samples were blotted and probed with the HPT coding sequence as described previously.

Undigested PH2 DNA only showed hybridization to the probe at the position of uncut DNA, demonstrating that the hygromycin gene is incorporated into high molecular weight DNA. The expected 1.05 Kb band for PH2 DNA digested with BamHI was observed, as had been shown previously. For PH2 DNA digested with PstI, a 5.9 Kb band would be expected if the hygromycin gene was present on an intact pHYGI1 plasmid. Two or more bands of variable size (size dependent on the position of flanking PstI sites within the host DNA) would be expected if the gene was incorporated into high molecular weight DNA. Two bands were observed with approximate molecular sizes of 6.0 and 3.0 Kb. This result is consistent with incorporation of the hygromycin gene into high molecular weight DNA. No hybridization was observed for DNA from control callus in any of the above treatments.

These results prove that the HPT coding sequence is not present in PH2 callus as intact pHYGI1 or as a small non-chromosomal plasmid. They are consistent with incorporation of the hygromycin gene into high molecular weight DNA.

VI. Plant Regeneration and Production of Seed

The line PH2, along with unselected control callus, were placed onto RM5 medium to regenerate plants as in Example I. After 16 days, the callus was transferred to R5 medium as in Example I. After 25 d on R5 medium, plantlets were transferred to R5 medium and grown up for 20 days. At this point, plantlets were transferred to vermiculite for one week and then transplanted into soil where they are being grown to sexual maturity.

EXAMPLE III

The procedure of Example II was repeated exactly except that different plasmids were used.

The plasmids pBII221 and pHYGI1 described in Example I were used as well as pMS533 which is a plasmid that contains the insecticidal *Bacillus thuringiensis* endotoxin (BT) gene fused in frame with the neomycin phosphotransferase (NPTII) gene. At a position 5' from the fusion gene are located segments of DNA from the CaMV and nopaline synthase promoters. At a position 3' from the fusion gene are segments of DNA derived from the tomato protease inhibitor I gene and the poly A region of the nopaline synthase gene.

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. Two tubes contained plasmids pHYGI1 and pMS533 and one tube contained plasmids pHYGI1 and pMS533 and one tube contained no plasmid but contained 5 µl TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done: 9×pHYGI1/pMS533 (potential positive treatment) and 2×TE prep (control treatment).

After bombardment, the callus from the pHYGI1/pMS533 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate. The same was done for one of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue (unselected control callus).

After 12 days, the callus on round 1 selection plates appeared to show about 90% of the weight gain of the unselected control callus All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin as ten 30 mg pieces per plate. After 22 days of selection on round 2 selection plates, the callus appeared completely uninhibited. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin.

At 74 days post-bombardment, a single viable sector was observed proliferating from the surrounding necrotic tissue. The callus line was from pHYGI1/pMS533 treated material and was designated 86R. The callus line 86R was transferred to F medium.

After 24 days, the callus line 86R had grown substantially. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin, and (ii) F media containing

60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin.

After 19 days of culture, the callus line 86R appeared to grow rapidly and was uninhibited on both levels of hygromycin. The control callus appeared to have only about 50% of the weight gain of 86R. The callus lines were transferred to fresh media at the same respective levels of hygromycin to further test the resistance of the callus line 86R. After 26 days of culture, the callus line 86R appeared uninhibited on 60 mg/l hygromycin.

Southern blots were performed on DNA isolated from the callus line 86R and control callus to confirm the presence of the hygromycin resistance gene and to determine whether the BT gene was present.

For detection of the HPT coding sequence, DNA isolated from 86R callus and control callus was digested with the restriction enzymes BamHI, XhoI, or PstI as described in Examples I and II. After hybridization with a probe prepared from the HPT coding sequence, the following bands were observed. For the BamHI digest, bands were observed at the expected size of 1.05 Kb as well as at approximately 3.0 and 2.3 Kb. This result demonstrates that the HPT coding sequence is present in the callus line 86R. The additional bands at 3.0 and 2.3 Kb indicate that either digestion was incomplete or that multiple rearranged copies are present. For the XhoI digest, a single band was observed at approximately 5.1 Kb. Because XhoI does not cut pHYGI1, this suggests incorporation of the hygromycin construct into DNA different than pHYGI1. For the PstI digestion, a large band was observed at approximately 5.1 Kb. This band appeared to be two fragments of similar molecular weight. Two or more bands would be expected from a PstI digestion if the gene was incorporated into high molecular weight DNA. In no case was hybridization observed for DNA from control callus for any of the above-mentioned digestions.

For detection of the BT gene, a Southern blot was carried out on DNA isolated from 86R and control callus digested with the enzymes BamHI and XhoI in combination. A BamHI, XhoI co-digestion liberates the 1.8 Kb BT coding sequence from the pMS533 construction used in this transformation. The blot prepared was hybridized to a probe prepared from the 1.8 Kb BT coding sequence. A band was observed for 86R DNA at the expected size of 1.8 Kb whereas no hybridization was observed for control DNA. Additional bands of much lesser intensity were also observed for 86R DNA. This result demonstrates that the BT coding sequence is present in the callus line 86R. This further demonstrates the introduction into maize of an unselected gene with potential commercial value. The name of callus line 86R was changed to CB1.

Plants are being regenerated from CB1 callus and control callus as described in Example I.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

What is claimed is:

1. A fertile transgenic *Zea mays* plant containing an isolated heterologous DNA construct encoding EPSP synthase wherein said DNA construct is expressed so that the plant exhibits resistance to normally toxic levels of glyphosate, wherein said resistance is not present in a *Zea mays* plant not containing said DNA construct, and wherein said DNA construct is transmitted through a complete normal sexual cycle of the transgenic plant to the progeny generation.

2. The transgenic plant of claim **1** wherein the heterologous DNA construct comprises a promoter.

3. A seed produced by the transgenic plant of claim **1** which comprises said heterologous DNA construct.

4. A progeny transgenic *Zea mays* plant derived from the transgenic plant of claim **1** wherein said progeny plant expresses said heterologous DNA construct so that the progeny plant exhibits said glyphosate tolerance.

5. A seed derived from the progeny plant of claim **4** wherein said seed comprises said heterologous DNA construct.

6. The transgenic plant of claim **1** wherein the plant is obtainable by a process comprising the steps of:

(i) bombarding intact regenerable *Zea mays* cells with microprojectiles coated with said heterologous DNA construct;

(ii) identifying or selecting a population of transformed cells; and

(iii) regenerating a fertile transgenic plant therefrom.

* * * * *

CIVIL COVER SHEET

The JS 44 civil cover sheet and the information contained herein neither replace nor supplement the filing and service of pleadings or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. (SEE INSTRUCTIONS ON THE REVERSE OF THE FORM.)

I. (a) PLAINTIFFS

SYNGENTA SEEDS, INC.,
GOLDEN HARVEST SEEDS, INC., AND
GARST SEED COMPANY,

(b) County Of Residence Of First Listed Plaintiff: (Except In U.S. Plaintiff Cases)

DEFENDANTS

DEKALB GENETICS CORPORATION

County Of Residence Of First Listed Defendant: (IN U.S. PLAINTIFF CASES ONLY)
NOTE: IN LAND CONDEMNATION CASES, USE THE LOCATION OF THE TRACT OF LAND INVOLVED

(c) Attorneys (Firm Name, Address, And Telephone Number)
John W. Shaw, Esquire (#3362)
Young Conaway Stargatt & Taylor, LLP
P.O. Box 391
Wilmington, DE 19899-0391
(302) 571-6672

Attorneys (If Known)

II. BASIS OF JURISDICTION (PLACE AN X IN ONE BOX ONLY)

- ☐ 1 U.S. Government Plaintiff
- ☒ 3 Federal Question (U.S. Government Not a Party)
- ☐ 2 U.S. Government Defendant
- ☐ 4 Diversity (Indicate Citizenship of Parties in Item III)

III. CITIZENSHIP OF PRINCIPAL PARTIES (Place An X In One Box For Plaintiff And One Box For Defendant) (For Diversity Cases Only)

	PTF	DEF		PTF	DEF
Citizen of This State	☐ 1	☐ 1	Incorporated or Principal Place of Business in This State	☐ 4	☐ 4
Citizen of Another State	☐ 2	☐ 2	Incorporated and Principal Place of Business in This State	☐ 5	☐ 5
Citizen or Subject of a Foreign Country	☐ 3	☐ 3	Foreign Nation	☐ 6	☐ 6

V. NATURE OF SUIT (Place An X In One Box Only)

CONTRACT
- ☐ 110 Insurance
- ☐ 120 Marine
- ☐ 130 Miller Act
- ☐ 140 Negotiable Instrument
- ☐ 150 Recovery of Overpayment & Enforcement of Judgment
- ☐ 151 Medicare Act
- ☐ 152 Recovery of Defaulted (Excl. Veterans)
- ☐ 153 Recovery of Overpayment of Veteran's Benefits
- ☐ 160 Stockholders' Suits
- ☐ 190 Other Contract
- ☐ 195 Contract Product Liability

REAL PROPERTY
- ☐ 210 Land Condemnation
- ☐ 220 Foreclosure
- ☐ 230 Rent Lease & Ejectment
- ☐ 240 Torts to Land
- ☐ 245 Tort Product Liability
- ☐ 290 All Other Real Property

TORTS

PERSONAL INJURY
- ☐ 310 Airplane
- ☐ 315 Airplane Product Liability
- ☐ 320 Assault, Libel & Slander
- ☐ 330 Federal Employers Liability
- ☐ 340 Marine
- ☐ 345 Marine Product Liability
- ☐ 350 Motor Vehicle
- ☐ 355 Motor Vehicle Product Liability
- ☐ 360 Other Personal Injury

PERSONAL INJURY
- ☐ 362 Personal Injury - Med Malpractice
- ☐ 365 Personal Injury - Product Liability
- ☐ 368 Asbestos Personal Injury Product Liability

PERSONAL ROPERTY
- ☐ 370 Other Fraud
- ☐ 371 Truth in Lending
- ☐ 380 Other Personal Property Damage
- ☐ 385 Property Damage Product Liability

CIVIL RIGHTS
- ☐ 441 Voting
- ☐ 442 Employment
- ☐ 443 Housing/ Accommodations
- ☐ 444 Welfare
- ☐ 440 Other Civil Rights

PRISONER PETITIONS
- ☐ 510 Motions to Vacate Sentence Habeas Corpus
- ☐ 530 General
- ☐ 535 Death Penalty
- ☐ 540 Mandamus & Other
- ☐ 550 Civil Rights
- ☐ 555 Prison Condition

FORFEITURE/PENALTY
- ☐ 610 Agriculture
- ☐ 620 Other Food & Drug
- ☐ 625 Drug Related Seizure of Property 21 U.S.C. 881
- ☐ 630 Liquor Laws
- ☐ 640 R R & Truck
- ☐ 650 Airline Regs
- ☐ 660 Occupational Safety/Health
- ☐ 690 Other

LABOR
- ☐ 710 Fair Labor Standards Act
- ☐ 720 Labor/Mgmt Relations
- ☐ 730 Labor/Mgmt. Reporting & Disclosure Act
- ☐ 740 Railway Labor Act
- ☐ 790 Other Labor Litigation
- ☐ 791 Empl Ret Inc Security Act

BANKRUPTCY
- ☐ 422 Appeal 28 U.S.C. 158
- ☐ 423 Withdrawal 28 U.S.C. 157

PROPERTY RIGHTS
- ☐ 820 Copyrights
- ☒ 830 Patent
- ☐ 840 Trademark

SOCIAL SECURITY
- ☐ 861 HIA (1395ff)
- ☐ 862 Black Lung (923)
- ☐ 863 DIWC/DIWW (405(g))
- ☐ 864 SSID Title XVI
- ☐ 865 RSI (405(g))

FEDERAL TAX SUITS
- ☐ 870 Taxes (U.S. Plaintiff or Defendant)
- ☐ 871 IRS - Third Party 26 U.S.C. 7609

OTHER STATUTES
- ☐ 400 State Reapportionment
- ☐ 410 Antitrust
- ☐ 430 Banks and Banking
- ☐ 450 Commerce/ICC Rates, etc.
- ☐ 460 Deportation
- ☐ 470 Racketeer Influenced and Corrupt Organizations
- ☐ 810 Selective Service
- ☐ 850 Securities/Commodities/ Exchange
- ☐ 875 Customer Challenge 12 U.S.C. 3410
- ☐ 891 Agricultural Acts
- ☐ 892 Economic Stabilization Act
- ☐ 893 Environmental Matters
- ☐ 894 Energy Allocation Act
- ☐ 895 Freedom of Information Act
- ☐ 900 Appeal of Fee Determination Under Equal Access to Justice
- ☐ 950 Constitutionality of State Statutes
- ☐ 890 Other Statutory Actions

IV. ORIGIN (PLACE AN "X" IN ONE BOX ONLY)

☒ 1 Original Proceeding ☐ 2 Removed from Court ☐ 3 Remanded from Appellate Court ☐ 4 Reinstated or Reopened ☐ 5 Transferred from another district (specify) ☐ 6 Multidistrict Litigation ☐ 7 Appeal to District Judge from Magistrate Judgment

VI. CAUSE OF ACTION

(CITE THE U.S. CIVIL STATUTE UNDER WHICH YOU ARE FILING AND WRITE BRIEF STATEMENT OF CAUSE DO NOT CITE JURISDICTIONAL STATUTSE UNLESS DIVERSITY.):
35 U.S.C. § 1 et seq.

Brief description of cause:
Cause of action for declaratory judgment of no patent infringement.

VII. REQUESTED IN COMPLAINT: ☐ CHECK IF THIS IS A CLASS ACTION UNDER F.R.C.P. 23 ☐ YES ☐ NO **DEMAND $**

Check YES only if demanded in complaint
JURY DEMAND: ☐YES ☒ NO

VIII. RELATED CASE(S) IF ANY (See instructions)

JUDGE: Chief Judge Sue L. Robinson
Chief Judge Sue L. Robinson

DOCKET NUMBERS: 04-305
05-535

DATE
10/5/06

SIGNATURE OF ATTORNEY OF RECORD
John W. Shaw

FOR OFFICE USE ONLY

RECEIPT # _____ AMOUNT _____ APPLYING IFP _____ JUDGE _____ MAG. JUDGE _____

INSTRUCTIONS FOR ATTORNEYS COMPLETING CIVIL COVER SHEET FORM JS-44

Authority For Civil Cover Sheet

The JS-44 civil cover sheet and the information contained herein neither replaces nor supplements the filings and service of pleading or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. Consequently a civil cover sheet is submitted to the Clerk of Court for each civil complaint filed. The attorney filing a case should complete the form as follows:

I. (a) Plaintiffs - Defendants. Enter names (last, first, middle initial) of plaintiff and defendant. If the plaintiff or defendant is a government agency, use only the full name or standard abbreviations. If the plaintiff or defendant is an official within a government agency, identify first the agency and then the official, giving both name and title.

(b) County of Residence. For each civil case filed, except U.S. plaintiff cases, enter the name of the county where the first listed plaintiff resides at the time of filing. In U.S. plaintiff cases, enter the name of the county in which the first listed defendant resides at the time of filing. (NOTE: In land condemnation cases, the county of residence of the "defendant" is the location of the tract of land involved).

(c) Attorneys. Enter firm name, address, telephone number, and attorney of record. If there are several attorneys, list them on an attachment, noting in this section "(see attachment)."

II. Jurisdiction. The basis of jurisdiction is set forth under Rule 8(a), F.R.C.P., which requires that jurisdictions be shown in pleadings. Place an "X" in one of the boxes. If there is more than one basis of jurisdiction, precedence is given in the order shown below.

United States plaintiff. (1) Jurisdiction is based on 28 U.S.C. 1345 and 1348. Suits by agencies and officers of the United States are included here.

United States defendant. (2) When the plaintiff is suing the United States, its officers or agencies, place an "X" in this box.

Federal question. (3) This refers to suits under 28 U.S.C. 1331, where jurisdiction arises under the Constitution of the United States, an amendment to the Constitution, an act of Congress or a treaty of the United States. In cases where the U.S. is a party, the U.S. plaintiff or defendant code takes precedence, and box 1 or 2 should be marked.

Diversity of citizenship. (4) This refers to suits under 28 U.S.C. 1332, where parties are citizens of different states. When Box 4 is checked, the citizenship of the different parties must be checked. (See Section III below; federal question actions take precedence over diversity cases.)

III. Residence (citizenship) of Principal Parties. This section of the JS-44 is to be completed if diversity of citizenship was indicated above. Mark this section for each principal party.

IV. Cause of Action. Report the civil statute directly related to the cause of action and give a brief description of the cause.

V. Nature of Suit. Place an "X" in the appropriate box. If the nature of suit cannot be determined, be sure the cause of action, in Section IV above, is sufficient to enable the deputy clerk or the statistical clerks in the Administrative Office to determine the nature of suit. If the cause fits more than one nature of suit, select the most definitive.

VI. Origin. Place an "X" in one of the seven boxes.

Original Proceedings. (1) Cases which originate in the United States district courts.

Removed from State Court. (2) Proceedings initiated in state courts may be removed to the district courts under Title 28 U.S.C. Section 1441. When the petition for removal is granted, check this box.

Remanded from Appellate Court. (3) Check this box for cases remanded to the district court for further action. Use the date of remand as the filing date.

Reinstated or Reopened. (4) Check this box for cases reinstated or reopened in the district court. Use the reopening date as the filing date.

Transferred from Another District. (5) For cases transferred under Title 28 U.S.C. Section 1404(a). Do not use this for within district transfers or multidistrict litigation transfers.

Multidistrict Litigation. (6) Check this box when a multidistrict case is transferred into the district under authority of title 28 U.S.C. Section 1407. When this box is checked, do not check (5) above.

Appeal to District Judge from Magistrate Judgment. (7) Check this box for an appeal from a magistrate's decision.

VII. Requested in Complaint. Class Action. Place an "X" in this box if you are filing a class action under Rule 23, F.R.Cv.P.

Demand. In this space enter the dollar amount (in thousands of dollars) being demanded or indicate other demand such as a preliminary injunction.

Jury Demand. Check the appropriate box to indicate whether or not a jury is being demanded.

VIII. Related Cases. This section of the JS-44 is used to reference relating pending cases if any. If there are related pending cases, insert the docket numbers and the corresponding judge names for such cases.

Date and Attorney Signature. Date and sign the civil cover sheet.

AO FORM 85 RECEIPT (REV. 9/04)

United States District Court for the District of Delaware

Civil Action No. 06-622

ACKNOWLEDGMENT OF RECEIPT FOR AO FORM 85

NOTICE OF AVAILABILITY OF A UNITED STATES MAGISTRATE JUDGE TO EXERCISE JURISDICTION

I HEREBY ACKNOWLEDGE RECEIPT OF 4 COPIES OF AO FORM 85.

10/5/06
(Date forms issued)

(Signature of Party or their Representative)

JEFFREY CASTELLANO
(Printed name of Party or their Representative)

Note: Completed receipt will be filed in the Civil Action